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Cuts all round for UK science

IN last week's *Nature* (page 565) we were editorialising about the growing weakness in support for R & D in the UK in comparison with other industrialised nations; this is not a weakness of resolve, it merely reflects a weak economy. The first budget of the new Conservative government underlines this weakness—expenditure in the higher education and science area is to be trimmed by between 1 and 1½ per cent as part of a very widespread operation to cut public expenditure.

The past two or three years have seen universities and research establishments pulling gradually away from the times of doubts and instability that characterised the mid-seventies. The University Grants Committee, charged with the responsibility for sustaining universities so that they have (among other things) 'well-found' laboratories in which research councils can support specific projects, has been able to make a little headway in replacing obsolete equipment; the UGC's equipment grant—of the order of £50 million—has even been growing in real terms by 10% annually. The research councils were pleasantly surprised late last year to pick up a promise from Mrs Shirley Williams, then Education Secretary, of £47 million additional cash over the next four years representing annual growth of around 3%. But now the prospects are not quite so bright.

The UGC has been successful in preventing the equipment grant from being cut (although universities will not be immune from the raising of VAT to 15%). But £9 million is being cut from the universities' recurrent grant and capital expenditure is also being trimmed. Nor is this all; there are several university wage settlements in the pipeline, and no guarantee that UGC will automatically receive the additional funds in full to pay for these settlements.

The research councils have had varied fortune. The Social Science Research Council has had nearly ten per cent of its funds taken away, the Agricultural Research Council next to nothing. The Science Research Council, by far the largest, loses £2.6 million of its budgeted £177 million for the present financial year, posing some threat to the 4.2 metre telescope now being planned for the new observatory in the Canaries.

The Medical Research Council might not seem to have come off too badly with a cut of just under 1% in its budget. It employs more people than any of the other research councils, however, and like the UGC will have to find money for increased salaries out of a reduced sum.

Scientists at the MRC units have been aware of this problem since early April when the previous government allowed a 5% increase in salaries in the unit's budgets for the 1979-80 financial year. Salary increases have been averaging at 15%, however, and many research units have been economising on new equipment whilst waiting to see what the new government had in store. With no increase in budgets for the units, the outlook was bleak; with a small cut it is bleaker still. Savings on equipment cannot continue indefinitely so posts will almost certainly be frozen and the already difficult problem of providing a reasonable career structure for scientists will be exacerbated.

It is clear that the cuts just announced do not represent any sort of detailed policy statement from the government on how it will pursue the support of science (with the possible exception of the social sciences). There is much nervous looking forward to the autumn, now thought to be the earliest time that the Department of Education and Science will reveal whether there are to be any major shifts in policy. There are relatively few straws in the wind, beyond that the government is involved in a 'quango' hunt at the moment. Quasi-autonomous non-governmental organisations (the UGC and research councils are amongst them) are said not to be terribly popular and there are moves afoot to trim them in various ways.

Those with memories of the way in which the Labour government of 1964 revamped the administration of science and technology will contrast the planning done by Labour in opposition with an apparent lack of preparation of science and technology policy by the Conservatives prior to this election, leading to a long period of waiting before policy surfaces. This is a disappointment; arguably science and technology policy is more important in 1979 than it was in 1964—even if there are few votes in it. □



US Defense Department destroys evidence of Vietnam devastation

Alastair Hay reports on a row in the US over the loss of photographs used in the much disputed NAS report on the effects of herbicides during the Vietnam war

PHOTOGRAPHIC evidence, used by the US National Academy of Sciences (NAS) for its 1974 report on the effects of herbicides in South Vietnam has been destroyed by the Department of Defense (DoD). According to Dr Thomas Dashiell, staff specialist for chemical technology at the DoD, the photographs were destroyed "in late 1977 through the spring of 1978".

Estimates of the acreage of forest sprayed with herbicides and the number of trees killed as a result of the spraying were based on these aerial photographs. Hundreds of rolls of film for the period 1958–1973 were examined by the Academy in order to assess the extent of the damage. Dr Philip Handler, president of the NAS, told *Nature* that most of the photographs were "taken by or in co-operation with the Department of Defense and were studied at the College of Forest Resources, University of Washington".

Dr James Bethel, Dean of the College of Forest Resources, was in charge of that study. His initial estimate of "merchantable timber" killed by herbicides was considered too low by a review panel and the figure was revised upwards before the NAS report was published. The report estimated that some 5×10^5 to 2×10^6 cubic metres of merchantable timber had been killed out of a total of 8.5×10^6 cubic metres sprayed. Spraying had in fact affected 10.3% of inland forests, 36.1% of mangrove forests, 3.2% of cultivated land and 5.5% of other vegetation.

Publication of these figures by the Academy created a storm of protest in scientific circles. Accusations that the estimates were too low were made by several scientists. Two members of the NAS committee reviewing the herbicide issue, Professor Paul Richards of the University College of North Wales and Professor Pham-Hoang Hô, University of Saigon, both dissented from the report on these grounds. Hô claimed that the estimates were out by an order of magnitude, a view which was supported by Professor Mathew Meselson of Harvard University and a member of the report's review panel. Meselson also claimed that the figures were 10 times too low.

The charges that the estimates were inaccurate were rejected by the NAS committee chairman, Professor Anton

Lang of Michigan State University. His reply to Hô's dissenting letter considered it "quite impossible" that the figures in the report could be out by the order of magnitude suggested.

Few NAS reports have created as much discord as this one. For this reason alone, many scientists argue that the evidence used by the NAS for the compilation of its report ought to have been kept. Dr Dashiell disagrees. He told *Nature* that the Department of Defense feels that "the NAS committee report represents a complete and well documented analysis of any ecological or environmental effects to the forests in South Vietnam". This, he says, was the principle reason for the study, and the photography provided for the committee study "represented only one means to reach their conclusions".

Dashiell says that the photography was kept for a longer period than originally envisaged. Following publication of the report, the photographs were placed in a repository at the University of Washington for use by scientists interested in doing further work on them.

This arrangement was approved by Dr Handler for the NAS, with the provision that a review be conducted after 12 months to determine whether the material be retained or destroyed. In the event, Dashiell points out, the material was left in the repository for two years, after which it was returned to the DoD where it remained for a further year before being destroyed. According to Dashiell, the scientific community "showed no interest during the lengthy time allowed for any review of their effort".

There is one scientist who was interested in these photographs and who is incensed about their destruction. Meselson, an outspoken critic of the NAS report, is in the process of compiling his own report about herbicide damage in Vietnam. In 1970, Meselson led a team of scientists to Vietnam on behalf of the American Association for the Advancement of Science (AAAS). Government officials in the White House, State and Defense Departments had advance warning of the team's preliminary report to the 1970 December meeting of the AAAS. A short time after this, an order was issued by the White House curtailing the use of



"It means 'Destroy Offensive Documents!'"

herbicides in Vietnam.

A summary of the AAAS findings were published in the Congressional Record on 3 March, 1972, but a final, fuller report of the team's investigations has never been written. Meselson has been chided in private on this point, but he told *Nature* that he hoped to be able to write the report this summer. One of the reasons for his delay was that he wanted to see the controversy surrounding the publication of the NAS report die down. This, he hoped, would give him an opportunity to produce his own report challenging the NAS estimates of timber damage on purely scientific grounds. Meselson makes no secret of the fact that the DoD's action in destroying the photographs has not made his job any easier.

Another reason advanced by the DoD justifying destruction of the photographs also seems rather lame. Dashiell insists that there were "physical storage problems" with the 35,000 items at the University of Washington repository so the decision was made to return the photography to the custody of the DoD. The story as related by Bethel at Washington University is slightly different. Bethel makes no reference to storage problems but says that, as no inquiries had been received about the photographs, he was "instructed by the Academy to return the photographs to the Department of Defense". According to Bethel, it was his understanding that the material was to be destroyed.

It seems, however, that although some probably irreplaceable scientific evidence has been destroyed, all is not lost; according to Dashiell, when the photographs were destroyed, the silver was recovered in accordance with "legislative mandates"! □

California set to cash in on British discovery

LAST month the Scripps Clinic and Research Foundation in La Jolla, California, announced that it is going into partnership with the pharmaceutical company Miles Laboratories to provide laboratory testing services and prepare immunochemical materials for research scientists and biochemical manufacturers.

The new company will have a staff of about a dozen scientists and will also be able to call on the services of over 150 scientists at Scripps on a consultancy basis. It will offer a range of testing services and products in immunology, endocrinology and toxicology. And one of its most active fields promises to be the rapidly-growing area of monoclonal antibodies. These are antibodies produced by a technique developed a few years ago by Dr Cesar Milstein and Dr George Kohler at the Medical Research Council's Molecular Biology Laboratory in Cambridge, UK. It allows antibodies tailored to identify specific antigens to be produced in relatively large quantities, avoiding many of the uncertainties involved in conventional techniques of antibody production from animal serum.

Already the availability of these techniques and the resulting antibodies has led to what one observer describes as a virtual explosion of interest by research scientists. This has been paralleled, almost simultaneously, by the growth of commercial interest in what promises to be a lucrative range of applications. But although the original research was funded by the British taxpayer, for one reason or another, the National Research Development Corporation did not take out early patents on the techniques and the UK may therefore have forfeited any royalty rights which, some observers believe, could eventually have totalled hundreds of thousands of pounds.

The technique which the Cambridge scientists developed involved fusing two cells, one—taken, for example, from the spleen of a mouse—that produces a range of antibodies (but would be relatively short-lived) and the other a rapidly reproducing tumour cell. The resulting hybridoma cells are separated according to the particular antibodies that they produce, and each cell strain can be cloned to produce a pure source of a single antibody.

For scientists, the use of monoclonal antibodies as a labelling technique (since each antibody will only recognise a particular molecule) is proving to have a wide variety of uses. These range from studies of the structure of cell membranes, to the search for viruses that may be associated with the growth of tumours.

Has Britain lost large potential royalties through a failure to recognise the commercial potential of antibodies?

David Dickson reports

Commercial interest in potential applications has been equally swift. The most immediate applications lie in the field of screening, for example in the early detection of cancer, or of foetal abnormalities (since foetal cells can be identified at an early stage in the mother's blood by looking out for genetic material from the father). Other uses range from tissue typing to assisting in the search for new vaccines.

Recognising the great potential of monoclonal antibodies, many large medical instrument and pharmaceutical manufacturers are now eager to enter the field. Some have been working on the techniques in their own laboratories; others—as with Miles Laboratories—by tapping into the expertise of the academic community.

"The type of work which we will be doing in this field will be dictated by the needs of the complex of companies under the Bayer (parent company of Miles) umbrella, for which we will carry out research on a contract basis," says Dr Ernest Tucker, director of Scripps' Immunology Reference Laboratory, which will be a division of the new company.

"These companies will come to us with specific needs for antibodies. And since we are an independent company, we will be able to carry out research at the request of other companies too. It is much more efficient to do it this way, since the support of basic research is very expensive."

As well as established companies, the applications of monoclonal antibodies is also attracting the interest of the venture capital market, a booming business in the US following recent changes in capital gains tax and security laws. Like recombinant DNA research, it is a field ripe for swift marriages between sophisticated science and ambitious entrepreneurship but the pay-offs seem at present to be much closer to fruition, while at the research level at least, the field is relatively free of federal regulations.

A typical creation of the entrepreneurial spirit is Hybritech Inc., a company set up in San Diego earlier this year by the west coast venture capital firm Kleiner and Perkins (which also provided much of the initial capital for the genetic engineering firms Cetus and Genentech).

Hybritech has already announced

plans for producing and marketing antibodies for detecting hepatitis, a first foray into a clinical diagnostic market where current sales, according to Hybritech president Howard F. Greene, are around \$200 million a year, and expanding rapidly. Mr Greene predicts that the return on investment for successful research could be as much as five times higher than that in the electronics field. "Already about 90% of the major companies in the diagnostic business have called us, expressing an interest in our work. They are all at various stages of looking at programmes in this area, but the feedback seems to be that the large corporations are not moving with the speed and flexibility made possible by venture capital operations," Mr Greene told *Nature* last week.

The growth of demand for monoclonal antibodies has caused its own logistical problems within the scientific community. Many laboratories offer the hybridoma cells that they have developed to other research workers in the field on request, but this is consuming a large amount of time and energy.

Pressure is now growing for a centralised store of characterised hybridomas. Already a number of cell centres, such as the cell distribution centre at the Salk Institute in San Diego, have a growing number of such cells on their lists. Some scientists are now calling for a new centre, possibly financed by the NIH, to store and distribute hybridomas.

"What seems to be needed is a central depository which would maintain cells that have been carefully characterised and presented by research workers, thawing them out occasionally to check that the characterisation has been maintained," says Dr Hilary Koprowski of the Wistar Institute in Philadelphia. "The time seems ripe for a conference of people interested in the organisation of such as depository, deciding what types of cultures should be accepted, how they should be characterised, and so on."

One problem that a depository would have to face, however, is how it should deal with requests from private companies. With substantial profits looming on the horizon—"Somebody is going to make money in the diagnostic business from this technique," says Jerome Goldstein, head of the Clinical Assays Division of Baxter Travenol Laboratories—licensing of hybridomas promises to become a lucrative business.

At Stanford University in California, scientists who have provided hybridomas to Salk's Cell Distribution Center have required that any research

workers requesting a sample of the cells sign a waiver promising not to use the cells, or the antibodies produced from them, for commercial advantage. The university itself is currently negotiating with a number of pharmaceutical and medical instrument manufacturers on terms for supplying them with specific hybridomas and antibodies developed in the university laboratories. Dr Neil Reimers, head of the university's technology licensing office, sees that as providing the university with a steady

stream of income under an arrangement agreed with the National Institutes of Health, which provided much of the funding for the original research.

Whether or not Dr Milstein's original work in Cambridge could have been patented is still hotly disputed. Some claim that, as the application of standard cell fusion techniques is in wide use around the world, there is nothing inherently patentable about Dr Milstein's use of the hybridoma (although novel hybrid cell lines result-

ing from the techniques can, it is generally agreed, be patented).

Others, however, feel that as a sufficiently novel application of the techniques, a patent might indeed have been granted, requiring royalties to be paid to anyone using the techniques for commercial purposes.

But the net result is that, like penicillin and other similar stories, monoclonal antibodies may join the list of the ones that got away from the UK. □

Italy's nuclear supremo goes for alternatives

The new chairman of Italy's nuclear energy committee plans to spend as much on alternative sources as nuclear power, writes **Robert Walgate**

FOR the first time in its history, the Italian National Committee for Nuclear Energy, CNEN, has received money to spend on the research development and promotion of alternative (non-nuclear) sources of energy. On 28 May, the outgoing coalition voted 5 billion lire (about £3 million) to CNEN to spend mainly on solar and wind power and on energy conservation.

The initiative is the work of CNEN's new Chairman, appointed on 1 February, Dr Umberto Colombo, previously research director of the firm Montedison. Colombo joined CNEN on condition that alternative sources would be included, and the 5 billion lire, he told *Nature* last week, is only the beginning.

"In Italy there has been no focal point for an energy policy" said Dr Colombo. "We want to become the focal point for all sources of energy which are alternatives to hydrocarbons."

At present, three ministers have partial responsibility for energy policy in Italy: the Minister for Industry supervises CNEN and the Ente Nazionale per l'Energia Elettrica (ENEL), which has responsibility for electricity supply; the Minister of Culture and Scientific Research directs the central body organising basic research in Italy, the Consiglio Nazionale delle Ricerche (CNR); and the Minister of State Participations has responsibility for the Ente Nazionale Idrocarburi (ENI) and the Istituto per la Ricostruzione Industriale (IRI); all of these being bodies with a more or less great interest in energy policy. The Minister for Industry has the largest responsibility—establishing the national energy



Colombo: "We might even be able to do without fast breeders"

plan, versions of which were published in 1975 and 1977.

"I want to make CNEN the entity responsible for all alternative sources, so that ENI remains responsible for fossil fuels, ENEL for electricity, and CNEN for R & D and promotion of nuclear and non-nuclear alternative sources, including the most important alternative—energy conservation."

The CNR is also undertaking an energy research project, but Colombo does not foresee conflict, rather co-ordination: "CNR is more involved universities; our contact is more with fundamental research and industry". ENEL and ENI have large geothermal projects "so we won't be able to do so much on that—probably we'll concentrate on hot rocks".

Dr Colombo hopes to make a substantial investment in non-nuclear alternative sources. The CNEN budget for 1979 is 200 billion lire (about £115 million), of which there is only 5 billion lire for alternatives; "but since we have only just started we could

hardly be expected to spend much this year". Next year, Colombo hopes to spend 30 billion lire (£17 million) on alternatives, and ultimately to reach an expenditure "very close to what we shall be spending on nuclear power".

Would it be possible to spend that much on new sources? "Well if you take some pioneer projects, and you consider that we are not interested so much in basic research as in demonstration units—in conservation, solar, wind, and so forth—it's possible". Clarifying the point, Dr Colombo said he has in mind an ultimate allocation of resources similar to that of the Department of Energy in the US, where nuclear—including fusion—still has more than other sources, but alternative sources have a substantial portion of the budget.

Nevertheless Colombo is convinced that nuclear power must make an increasing contribution to Italy's energy supply; at present one major and three minor reactors supply 0.7% of Italy's total energy needs and 2.7% of its electricity. By the year 2000, Colombo would like to see 20% of Italy's energy needs supplied by nuclear reactors requiring a building rate of two reactors per year; solar power, the main alternative contributor, might supply 7% by that date, he estimates.

"We do not have North Sea oil, we do not have the gigantic coal fields you have in the UK; at present we are very dependent on imported hydrocarbons. 85% of our energy is supplied by hydrocarbons, and 76% is imported energy. This dependence is not acceptable; we are very vulnerable. Our oil bill may go up this year, because of the price increases, by £1 billion; that's equivalent to exporting another million automobiles, if we want to keep our balance of payments in order . . . so a nuclear programme, as well as a vigorous programme on alternatives, is necessary for Italy."

Colombo makes a fairly classical connection between energy consump-

tion and economic growth. After conservation measures have been applied, he estimates Italy will be consuming 40% more energy in the year 2000 than now. "Don't measure that against UK standards. Our per capita consumption is 2.4 tonnes of oil equivalent; you have 3.7; so we must make great progress to reach other European countries. We can't possibly stay much behind them if we want to develop our own industry." The 40% increase corresponds to an annual rate of about 1.4%, and is based on an assumption of economic growth of 2.5% per year with an 'energy elasticity' (ratio between energy and economic growth rates) of some 50%.

Colombo is in the process of constructing his first 5-year plan for CNEN. "In the past everyone has been very superficial: both parliament and government, and CNEN, ENEL and the industry." Now Colombo believes that "we at CNEN must express our opinions forcefully, justify them with numbers and facts, and submit our ideas to a discussion in parliament." Then it is a matter of responding to the political will of parliament and government. Colombo hopes to submit his plan by the end of the summer.

Previously CNEN's energy policy was "very much in the air, something rather abstract". CNEN was moving in many different directions, in each of them "below the necessary threshold" of potential, manpower, and capital. Furthermore CNEN has been hampered by the government's inability to define a politically and publically acceptable nuclear plan. But in any case the previous plan "was too ambitious, and not credible at all".

"There were gigantic plans to build up a huge number of power stations, but the plans were never implemented. . . . They required almost a menu of different nuclear technologies, which—particularly after the Harrisburg incident—is no longer advisable. We need to concentrate on one type of proven reactor, and to put a great emphasis on the safety and security aspects of that one. I therefore will use my influence and my power—as far as possible—to determine this concentration of effort on one type of reactor. I do not think we have the technical or organisational capability to go ahead in many different directions in nuclear power in Italy."

The reactor will probably be the Westinghouse pressurised water reactor, which has also been chosen in France and Germany; this not because Colombo has any particular attachment to that but because the concentration of international effort and experience on it should make it one of the safest systems.

Nevertheless the Italian industry will continue to work on other reactor

systems, like the Tirrhenia heavy water reactor presently under construction "to enable the industry to work on the commercialisation of CANDU-type reactors throughout the world". CNEN will also do direct and sponsored research and development of fast breeders and fusion reactors.

On nuclear safety, CNEN is at present both promoter and regulator of nuclear power—an unsatisfactory situation which Colombo expects to be changed sometime next year. At present "they both depend on me" says Colombo. But for the moment he is reluctant to give up the dual role. "We must first make the safety division of CNEN stronger technologically; I want to fortify it a little bit before giving it to somebody else. Secondly, the safety problem in nuclear plants is not just a health problem, it's a matter of checking the design, construction, and operation of very complex technological plants, which requires some technical expertise. If safety is left in the hands of medical people alone, and epidemiologists, they will cover very well the biological part but won't be competent in the other. So we need both, and we need to move towards the creation of an independent organisation. I hope it will be constituted under the premier, who controls both industry and health, allowing the organisation to include both types of competence".

"I am grateful to those who have been against nuclear power, although I do not share their ideas" said Colombo. "They have brought the problem to the surface, and obliged us to be more frank and serious in our considerations." In the past, he said, the nuclear authorities in Italy have been too arrogant in their dealings with the public; it is time to consider clearly and openly all the risks, both with and without nuclear power.

Furthermore, "if we are able to develop alternative sources immediately so that they can penetrate by 7% by the year 2000, and take off thereafter, we might even be able to do without fast breeders. But if the rate of penetration is slow, and if nuclear fission is the bridging energy source up to 2020–2030, we will have serious problems with uranium."

Colombo therefore wants to keep fast breeders open as an option, and Italy is already building pilot reprocessing plants "so that when we have to decide, we can decide from a strong position".

Ultimately, however, it is all up to the Italian government. "I'm powerful as far as propositions are concerned" says Colombo "but I'm not the one who decides". Colombo will be submitting his first 5-year plan to the government "in a month or two". □

Energy efforts should shift from research to conservation

THE US Administration is placing too much emphasis on research into new energy technologies, and not enough into conservation techniques even though the latter have already produced considerable energy savings, according to a paper published by the Union of Concerned Scientists.

Dr Vince Taylor, the author of the paper, points out that improvements in efficiency made by industry since 1973 have contributed twice as much as oil obtained through the Alaska pipeline to the energy needs of the US last year. "90% or more of the solution to our energy problems will come from improvement in energy productivity—10% or less from supply extension. Yet the administrative and budgetary resources are being allocated in just the opposite proportions," Dr Taylor writes.

Dr Taylor lays out what he calls an 'easy path' strategy which, he claims, would redress imbalances in the energy budget by concentrating on factors such as improving the efficiency of motor vehicles and of energy use in domestic and commercial buildings, and selective switching between fuels. This strategy, he says, does not depend for its success on expensive and uncertain long-range R & D projects. □

Carter proposes new fund to clear up chemical wastes

PRESIDENT Carter has submitted to Congress legislation setting up a \$1.6 billion fund for cleaning up hazardous chemical waste dumps and the effects of chemical and oil spills, in what one aide has referred to as "the most important environmental legislation" to be proposed by the Administration in the current year.

The President's move has been prompted both by general concerns about the effects of industrial pollution, and more specifically by a number of recent highly-publicised cases in which the previous dumping of toxic wastes has led to serious environmental and health hazards.

Against the advice of a number of federal agencies (although not the Environmental Protection Agency) the President has also decided that the industries responsible for the wastes should carry a large part of the bill for clearing them up. Consequently 80% of the fund would come through a series of fees imposed on oil refiners and chemical manufacturers. □

Whitehall urged to relax grip on research funds

THE UK Agricultural Research Council is now poised to gain control of £2½ million of its funds previously tied to work commissioned by the Ministry of Agriculture, Fisheries and Food (MAFF). And this desire for greater freedom from the commitments of the Rothschild reorganisation of research has been reinforced by a plea from the Medical Research Council that Department of Health and Social Security control of its funds be reduced.

Both developments were revealed following last week's publication of the minutes of meetings of the House of Commons public accounts committee. In the case of the ARC it was stated that a review had been carried out by the MAFF and the Department of Education and Science under the previous government and this had investigated charges made by the ARC for use of its facilities. In particular, it was felt that more money should be paid by the MAFF for superannuation of staff carrying out its work and also for some capital costs. The full charges amounted to about £2½ million and it is now known that the Ministry has decided to pay this figure.

Although the MAFF will still control 54% of the ARC's budget, which last year totalled £45.6 million, it will now find £2½ million of its money earmarked for staff and other costs. This will free an equal sum for direct use by the council, which is likely to spend it on priority areas such as the genetic manipulation of plants with the aim of introducing new characteristics in crops.

However, the agreement to charge full economic costing for ARC facilities still has to be confirmed by the new Conservative administration, and this decision is expected shortly. Even when agreed, the effect will not be immediate, as many MAFF projects are being funded over long periods and there is no question of them being abandoned. But in the long term this financial reallocation will certainly give the ARC far greater flexibility in its undertakings.

In his plea to the committee, Dr James Gowans, the secretary of the MRC, said the transfer of its funds to DHSS control represented a vulnerable area in the council's £60 million budget: "It is vulnerable because the rules say the government departments are expected to spend the transferred funds with particular councils, but they do not have to."

He described the present arrangement as not being ideal—a dissatisfaction that stems, in part, from the 1976 cutbacks which resulted in the DHSS suddenly reducing its funding of

the council by 10%. "I should like to enter a plea that the MRC feels a little uneasy about the large chunks of its funds which could with political change be either decreased or might conceivably disappear. In fact, I should like to see in the long term the fraction of my council's total money, which is free money, increase—to give us greater stability and flexibility", he told the committee.

Dr Gowans said there were particular difficulties in trying to direct biomedical research through departmental contracts. One problem stemmed from the MRC's need to supervise all research, from basic to applied.

"In cancer, for example, we would



James Gowans, MRC: "vulnerable"

consider it essential to start with basic molecular biology and go right through to trials of new chemicals and radiation therapies", he added. It was of no use to pick a small fraction of a programme and concentrate only on that aspect.

"The other point is that if one looks back at the history of medical discovery, one finds that the key discoveries have all been made by accident", he stated. This also made it difficult to commission effective pieces of research and Dr Gowans indicated that he would prefer a system of saturating the very best talent with funds, although this would be hard to justify in times of financial stringency when many workers were competing for limited funds.

However, in his report to the committee, Sir James Hamilton, permanent secretary at the Department of Education and Science (which controls the major portion of the MRC's budget) said it would be wrong to suggest the idea of the customer-contractor relationship had failed—although he revealed that the DHSS-MRC relationship will be reviewed this autumn. This is to ensure basic objectives are being met in terms of scientific and financial accountability and are not being unnecessarily burdensome in bureaucratic terms.

Robin McKie

WHO lays down safety guidelines

THE World Health Organisation is making progress with its attempt to lay down guidelines on occupational exposure to toxic substances. A meeting in Geneva this month agreed on a methodology for arriving at permissible levels of exposure to four heavy metals—cadmium, lead, manganese and mercury.

The first step is to examine all the available medical and scientific information, followed by the decision on what degree of risk can be tolerated. Degree of risk has to be decided at national level, partly because circumstances differ in some countries. For example, in the tropics, industrial workers may also be exposed to endemic diseases affecting the kidneys, liver or lungs. The acceptable level of metals toxicity affecting the same organs would therefore have to be much lower there than in northern industrialised countries.

The meeting made a breakthrough with agreement between the US and USSR on what should be the actual basis for exposure. The Soviets are considering switching to the American system of time-weighted exposure and abandoning their previous "maximum exposure" basis. There has also been

agreement on the principle of adverse health effects" for the four metals under consideration. Two criteria have been laid down: (a) is the effect reversible or not? (b) are compensatory mechanisms in the body impaired? From this it is possible to define the effects of each substance on an agreed "target organ".

Finally, the Geneva meeting agreed on permissible levels for each metal, both "biological levels" in the person affected and atmospheric levels in the place of work or in the immediate vicinity of a plant such as a lead smelter.

The need for guidelines such as these was because of the wide discrepancies in permissible levels between various industrialised countries and the feeling that guidance would help Third World countries moving into industrialisation. Previous attempts to recommend levels of toxicity through the International Labour Organisation have tended to be blocked by employers pressure groups. The WHO approach was specifically medical and scientific, but it is understood there were, nonetheless, messages of strong disapproval from a number of industrial firms.

Peter Collins

news in brief

Sussex students occupy physics building: The protest at Sussex University (14 June, page 568) continued to escalate as students occupied the physics building, which houses the university's main telephone exchange. The telephone service to the university was cut off. The occupation, which began on 15 June and is expected to last for at least another week, is the result of a week's failed negotiations. Students elected the "excluded" student body president Richard Flint and engineering student Shaun Fensom, to present their proposals for joint talks with staff at the university Senate meeting. (Their proposal has been widened to include the case of 305 students threatened with academic sanctions because of a current rent strike against university housing. The students voted that sanctions or threats of sanctions should be withdrawn as a precondition for talks and voted to go into occupation if the amnesty proposal was rejected.

Upon seeing the two excluded students in the meeting hall, Vice-chancellor Denys Wilkinson called off the Senate meeting but was persuaded by moderate staff to reconvene it. A morning session was followed by a massive rejection in the afternoon of the student proposal. There are no immediate plans to call off the occupation and administrative hopes that the summer vacation would dampen opposition were dashed as the students voted to disrupt conferences during the summer at their last general meeting on 19 June.

Philippines call halt to nuclear plant: The Philippine government has halted construction work on a £600m Westinghouse reactor for alleged violations of the safety warranty. The plant was begun in 1976 and was to be finished in 1983. The government acted when Westinghouse refused to send a safety team to the Philippines to discuss the Three Mile Island accident, and after the US Union of Concerned Scientists had sent a detailed plan of deficiencies in engineering and design. Westinghouse had commissioned an independent inquiry which found that there was no evidence that the reactor would be unsafe. The ban on building will not be lifted until the government is convinced that the plant posed "no danger to the people."

Vietnam veterans studied for herbicide exposure: Vietnam veterans are to be the subject of a study of the long term effects of exposure to the herbicide, 2,4,5-T. The investigation, ordered by the Carter administration, will examine 1,200 veterans exposed to the herbicide during spraying missions over Vietnam in the years 1962-1970. Scheduled to last six years, the investigation will be carried out by the Air Force and the Department of Health, Education and Welfare; it will supplement an inquiry already begun by the Veterans Administration. Concern about the health hazards of 2,4,5-T and its tetrachlorodibenzo-p-dioxin contaminant—a known teratogen and carcinogen—has increased in recent years. Vietnam veterans were exposed to the herbicide in a formulation called Agent Orange of which 2,4,5-T is one of two components. Agent Orange was known to have much higher concentrations of the dioxin than 2,4,5-T preparations in current use.

Clinical data from the 1,200 subjects will be compared with that from a control group of 1,800. Doubts still remain about the scientific methods to be used in the study. Evaluation of the results will also prove difficult with the wide ranging clinical symptoms attributed to exposure to 2,4,5-T. Veterans are claiming that psychological disturbances, malaise and death from cancer can all be traced to exposure to 2,4,5-T.—*from Alastair Hay.*



New HSE microbiological adviser to be tough on safety: The UK Health and Safety Executive has appointed Dr Bob Harris (left) as its adviser on microbiological hazards at work. Harris, director of the former Microbiological Research Establishment at Porton Down, is a firm believer that society must control the safety of research work. He also supports trade union and general public representation on committees such as the

Genetic Manipulation Advisory Committee. Harris took up his post of senior consultant adviser on 21 May. He will oversee the development of contaminant assays and training of HSE staff in their use, and he will represent the HSE on committees and public bodies. Harris is an experienced cancer researcher, most recently as head of the Imperial Cancer Research Fund's Division of Experimental Biology and Virology. His appointment is expected to silence criticism from "a minority of researchers" who have opposed HSE interventions in laboratory safety.

British delegation tries to sell Magnox reactors in Peking:

A British Energy Exhibition organised by the UK Department of Energy and the British Overseas Trade Board visited Peking from 6-16 June to sell energy technology to the Chinese. British Nuclear Fuels Ltd and the Nuclear Power Company are trying to interest the Chinese in the UK's Magnox reactor, an old first generation machine that is considered to be the safest nuclear reactor. Mr Robert O'Neil, head of engineering safety and technology at the UK Atomic Energy Authority, gave a long talk on reactor safety to a Peking conference on 12 June. O'Neil told the Peking audience that the probability of receiving a lethal dose of radiation at a distance of 15 km from a reactor was approximately the same as being hit by a meteorite or "killed in a supernova explosion of a star". There is no record yet of specific questions asked by the Chinese about reactor safety.

Environmental agency urges destruction of nerve gas bombs:

The US Environmental Protection Agency has recommended to the Defense Department that it should destroy the Army's stock of 900 Weteve nerve gas bombs, rather than move them from a Denver arsenal to a base in Utah. This follows evidence that a number of bombs are leaking inside their sealed containers.

Although the Army had initially announced its own plans to destroy the weapons (the most modern chemical weapons in the US armoury produced shortly before a ban was imposed by President Nixon in 1969). Defense Secretary Harold Brown announced last year that the weapons would be kept as a deterrent to any use of lethal gas in war by the Soviet Union. Plans to move the bombs, which contain more than 300,000 pounds of gas, were first proposed for last year, but postponed when two were found to be leaking. Following a new announcement last month by the Defense Department that it would shortly begin transporting the bombs after an investigation had concluded that the rest of the bombs were safe and could be moved, Utah governor Scott Matheson announced his intention to seek a court order preventing their transport.

In a letter to the officials of the Rocky Mountain Arsenal, where the bombs are currently housed, the EPA says that, in view of newly-discovered leaks, the bombs should be destroyed.

CERN—the next 25 years

The European centre for subnuclear physics—CERN—celebrates its 25th anniversary next week. But it is a time for Europe to look forward, not back. **Robert Walgate** reports

AMERICAN high energy physics laboratories—there are three major ones—have generated one or two Nobel prizes, and much spectacular physics. On the other hand CERN, the European centre for high energy physics, has discovered 'neutral currents' (a new kind of weak interaction), but nothing else with the feel of a Nobel prize. (The one for neutral currents hasn't been awarded yet.) CERN has been much praised for the quality of its work; but sometimes this has felt like being patted on the back for trying hard. However, all this seems about to change.

Burton Richer, an American who won a Nobel prize for discovering the J/ψ (indirectly a discovery of the charmed quark) agreed last week that in the last 25 years the greatest discoveries have been American; but in the next 25, he said, "Europe will become the senior partner". And according to Leon Lederman, also a man of spectacular discoveries (he recently found the next quark, bottom, in a 9.5 GeV particle called ψ), "the US is going to have a rather hard time competing".

However "it's nonsense" says Lederman, to say that CERN was too concerned with quality and missed the spectacular results. "Anyone can get spectacular results". The reason the US got most of the big discoveries was partly the statistics of small numbers—luck. "Plus we had a heritage of experience. We had accelerators running in 1951." CERN's first, the SC, started up in 1957. And now he envies the quality of CERN engineers. "The European engineering education is better based in mathematics and physics and ideally suited to accelerators."

Lederman should know, for it is, in some sense, his job to compete: he has been appointed director of The Fermi National Accelerator Laboratory (Fermilab) in Chicago, America's nearest equivalent to CERN. But he says "we're undermanned by a factor of 2 or 3; we don't have the people or the money". The previous director, Robert Wilson, resigned over a question of funding.

But CERN isn't going ahead simply because the US is falling behind. It has three adventurous plans on the books: one approved and building; one on the verge of approval; and one very ambitious scheme yet to be put to the political test.

The first (the antiproton collider) should generate physics from the collisions of protons and antiprotons within the present 500 GeV accelerator ring towards the end of 1981. There's probably a Nobel prize in it through the discovery of the 'intermediate vector boson' (a particle as important to modern physics as the structure of DNA was to 1950s biology). The second (LEAR) should use stored antiprotons for unique experiments in low energy nuclear physics. And the third (LEP) would generate 200 GeV collisions between electrons and positrons (presently the cleanest way of investigating the fine structure of matter) towards the end of next decade. There are probably a few Nobel prizes in that.

A firm and unanimous proposal for LEP, the result of the deliberations of hundreds of physicists throughout Europe in the technical panels of ECFA (the European Committee for Future Accelerators), should go to CERN Council on 9 November this year. LEP is CERN's future for the late 1980s and 1990s; it is up to governments to decide if CERN can have it. (The clash between Germany and CERN over the siting of LEP has died down; CERN will have it, while Germany's national Hamburg laboratory DESY will add protons to its electron rings: at least that's the physicists' plan.)

Hamburg, too, is ahead; experiments are underway there on what is presently the world's highest energy electron-positron machine, PETRA, with the American equivalent, PEP (Burton Richter's machine), coming on in November about a year later because of funding delays.

But America hasn't given up. According to Lederman "I think Fermilab is going to cause CERN a lot of grief". Fermilab is building—and has been building for some time—a ring of superconducting magnets (the 'energy doubler') to join the normal magnets in its 500 GeV proton accelerator tunnel, and these magnets will give Fermilab the capability of reaching beam energies of 1000 GeV to collide with a target, or making collisions between 1000 GeV and 1000 GeV countermoving protons. Lederman hopes to phase in physics on the colliding beams by 1982; and on fixed target by 1983. This would give Fermilab the highest energies in the world. "The doubler is the only way



Leon Van Hove, CERN research director: leaving "after a very interesting period of defining future projects"

we can get ahead" says Lederman.

(Ultimately the USSR should overtake Fermilab with UNK, a 10,000 GeV—10 TeV—machine.)

Furthermore at Hamburg there have been problems in reaching design collision rates (which determine whether an experiment can be done in months or years) because of a complicated acceleration system which will not trouble PEP. Richter says "I've laid a number of bets that within two weeks of first injection we will be at 10% of design luminosity". PETRA has been sitting around 1%, and although this figure is rising fast there will be a lot of physics left for PEP to do.

A few years ago such competitiveness was justified in terms of repeating experiments: if Fermilab discovered something, then CERN ought to check it. There was an example of that recently, when an anomaly in neutrino interactions discovered in a rough and ready fashion at Fermilab was checked—and discounted—in a more sophisticated experiment at CERN.

Sharing unique facilities

But the future seems to be one of unique, continental facilities and no overlap.

So a number of physicists—particularly American ones—foresee a period when there will have to be increased international cooperation, even going so far as a transatlantic pooling of funds on projects such as, for example, the energy doubler at Fermilab. But according to the chairman of ECFA, Marcel Vivargent, this is impossible at present; CERN funds are very rigidly controlled by the member states and must be spent only for Europe. "But if LEP is accepted"

said Vivargent last week "we will have to discuss cooperation".

Leon Lederman, Fermilab director, raises an interesting issue: there are 26 European groups currently working at Fermilab, he says, who have come through the usual selection procedure by merit. "We have no way of assessing where a proposal comes from . . . but the energy doubler is going to raise a question of discrimination." With their (disputed) better funding the European groups will have better equipment for their experiments, he says, and without discrimination this would leave the Americans in the cold. There seems to be a possibility here that if competition does not ease, it will increase. No sharing of funds, no sharing of experiments, may be a new threat from the US to Europe.

Leon Van Hove, research director-general of CERN, feels that the normal exchange of groups (evidenced by the 26 European groups at Fermilab) will suffice; and that although exchange is increasing, no new procedure or institution (to match groups and laboratories) is necessary. The International Committee for the Future of Accelerators (ICFA) under the International Council of Scientific Unions (ICSU) would be sufficient to discuss the matter, he feels.

Burton Richter, on the other hand, believes that the old ways will not suffice, because of the large and increasing cost of the apparatus that must be built up around an accelerator to do a particular experiment. A single detector at LEP would cost about \$20 million, he estimates; who is going to pay for the apparatus, the group or the laboratory?

And how should the operating costs of the accelerators be divided? According to Lederman, Fermilab's electricity bill is presently \$7 million, and may rise to \$10-12 million next year (though the superconducting energy doubler, used not to double beam energy but to save accelerator power, could cut the costs 70%). And LEP, according to some estimates, if it used conventional magnets, could consume 500 MW of electrical power. (But that estimate is sensitive to the assumed radius, and to the energy of the machine. The consumption rises as the fourth power of the beam energy.) With costs and consumptions such as these, intercontinental cost-sharing may be necessary.

Whatever agreements may prove to be necessary on this level, there is a definite feeling both in Europe and the US that Europe now has the initiative. In the short term the US has PEP, which will be competitive with PETRA; in the long term, the American future depends on the

technology of superconducting magnets, which Fermilab is still wrestling with. (Another US machine for the mid-80s, Isabelle, a high energy East Coast machine to collide protons with protons, also needs superconducting magnets.) Lederman says Fermilab is about to go into a 1,000 magnet production run (they have produced 200 in various batches so far) but CERN cynics say they have heard that before; the technology is proving very difficult.

(If LEP needs superconducting accelerating cavities to save power costs and reduce that 500 MW to something manageable, it will face a problem of even greater magnitude. But test cavities are already in construction at Karlsruhe in West Germany for trials at DESY.)

Is there a simple reason why Europe appears to have taken the lead? According to Lederman, funding has a lot to do with it. "In the early 50s we were better funded, but somewhere in the 70s the budgets crossed." In CERN, it is not felt that Europe has much more money to spend—rather that the US is stretching its resources across too many projects. "If we had the energy doubler, PEP, and Isabelle, I wouldn't say we badly off" said one CERN physicist. And according to Leon Van Hove "to run three big labs on a small budget is trying to square the circle".

Delayed advantage

Also, the delay in building the 500 GeV super proton synchrotron (the SPS) at CERN may, in retrospect, have been an advantage. While the US committed a large fraction of its funds to the construction of Isabelle, which many now see to be an outdated machine, it became clear that a major way forward in physics was to collide particles with their antiparticles (electrons with positrons, which gave us charm, or protons with antiprotons). Hence when the SPS was built, European physicists were free to use their new experience in allocating the potential European budget to the antiproton collider and to LEP.

Furthermore there appears to be a new mood in CERN. It used to be accused of being very strong on engineering—accelerator design—but unadventurous on physics. There was a strong division between the accelerator people who wanted a beautiful machine, and the users who wanted physics quickly. Now that divide is being bridged, and the accelerators are really being designed to suit the physicists, while the physicists are becoming more aware of the real constraints of design. LEP is certainly benefiting from this, and so is the antiproton collider.

That particular project is very instructive. An Italian with Italian temperament and American determination, Carlo Rubbia, who had been doing neutrino experiments at Fermilab and making a few discoveries (dimuons, events now connected with charm, and the anomaly later discounted at CERN), decided that the Fermilab and CERN accelerators could be used to make the intermediate vector boson (this latest holy grail of physics) if antiprotons could be injected into them the wrong way round. Simple in principle, but difficult in practice; but Rubbia was enough of an accelerator engineer to make some sensible proposals. He made them, to CERN and Fermilab, around 1976, and from then on the labs were in competition.

Then a number of interesting events occurred. First, it became clear that the vacuum in the Fermilab machine, at 10^{-7} Torr, while good enough for the accelerator, would not do for day-long storage of intense beams (beams were lost in 30 minutes); while the SPS, over-designed to some American tastes at 10^{-8} Torr and in easy reach of 10^{-9} Torr, could do the job it had not been designed for.

Second, the CERN team in charge of future projects rejected the scheme, which involve creating antiprotons, catching them, and 'cooling' them into a confined beam, as impractical—so the project was taken on directly by people who were in favour of it. "There were people at CERN who were determined not to be beaten again" said one member of Rubbia's group. "They'd lost the J/psi and the upsilon to the US and they were not going to lose the IVB". There was, in fact, a kind of breakthrough by radical elements, greatly assisted by the persuasive powers of the antiproton advocate, Carlo Rubbia.

Third, the CERN directorate swung wholeheartedly behind the scheme (in fact they had a choice between two radical proposals, one involving electrons; it's arguable which was best) and put a good deal of CERN money into it.

And finally, the CERN accelerator physicists and engineers moved rapidly in reaching the solution of a very difficult technical problem, and proving it in a beautiful experiment, the initial cooling experiment (ICE). The project drew on all available CERN expertise, but decisions were taken sequentially as step after step was proved. There are still two years and many problems to go, but the antiproton projects appears to be CERN at its very best—using not only its skills, but also a large dash of imagination. Yes, Professor Lederman, it's going to be a very difficult combination to beat. □

Science in Bolivia: the great divide

Bolivia has had 190 Presidents in 150 years of independence. Seven out of 10 of its poverty-stricken people are illiterate, and yet the country has eight 'liberal' universities for the privileged few, generally those of European descent.

This frustrating formula is carried over into science: research is confused, geared to foreign backers and largely unrelated to the needs of the people.

John Hutton, from the Department of Clinical Biochemistry at Addenbrooke's Hospital, Cambridge, visited Bolivia on a Nature writing fellowship.

THE village of Patacamaya lies to one side of the road that links the commercial centre of Bolivia, La Paz, with the mining town of Oruro. To the outsider, it is just like any other of the windswept settlements scattered across the Altiplano, the high plateau where 60% of Bolivia's five million people live. The village square seems deserted, the adobe huts practically uninhabited. There is a pervasive air of forlornness, only dispelled when the first light appears on a Saturday. Before dawn, trucks that have arrived from all over the countryside crowd into the marketplace, stacked with produce and people. Salt blocks conveyed by llama trains from the salars to the south west are exchanged for fresh fruit and vegetables transported across the mountains from the jungle regions to the east. What money changes hands is spent on a few manufactured goods from La Paz or a farming implement forged in the open air foundry beneath the eyes of the crowd. In the late morning it is time to celebrate the purchase of a cow, perhaps, or consult with a herbalist, but by the afternoon the dust has settled and the stillness has returned.

Less than seven kilometres from the village, and in stark contrast, is a Ministry of Agriculture research station, a monument to the agricultural potential of the high plateau. Established on what was formerly an estate thought too poor for redistribution under the agrarian reform of 1953, it demonstrates the transformation that can be wrought by the application of machinery, fertilisers, and plant and animal breeding techniques. To the Indian farmer tracing a furrow behind a single-bladed wooden plough tethered to a pair of oxen or to the shepherd following his few llamas and sheep as

they seek sustenance amid the stones and stubble nearby, the research station seems no more of his world than is the Russian satellite tracking station also located at Patacamaya. If anything, it only serves to remind him that he stands outside the money economy, that nobody cares for his education, that some of his children will die before reaching adulthood and that none of his family can expect to live for more than fifty years. Although the revolution has given the native farmer back his land, it seems that the administration has no place for him. With respect to health and education, the rural dweller is ignored, left to eke out a subsistence living from an inhospitable environment with little but his own meagre resources.

Somewhere between the agronomist of the research station and the subsistence farmer there is another man: a farmer's son, perhaps, whose primary education at the local school sets him apart, and who sees no future for himself in the bleak countryside. Given the opportunity, he would make the journey to La Paz, in the hope of becoming a gardener or a servant, or to work in one of the few factories. In time he might hope to own a truck or to rent a stall in the sprawling marketplace, and his dreams would be to change his traditional habits, purchase a few goods such as a radio or television and to educate his children into a better place in society.

The present Bolivian educational system, which leaves 70% of the population illiterate, still finds a place for eight universities—despite the country's small population. Amongst them is the oldest university on the South American mainland, the Royal and Pontifical Higher University of San Francisco Xavier of Chuquisaca. Traditionally, these institutions served to maintain the Hispanic cultural heritage and they were dedicated to the



formation of a liberal professional elite, whose ranks excluded both women and the native population.

Today, amalgamated under the title of the Bolivian University, they are nominally non-sectarian, academically autonomous and open to all. Despite this, membership has generally remained the privilege of an urban class of European or mixed European extraction. Enrolments in economics, law, medicine and engineering still account for more than half the student population, and so the country has too many doctors and too many planners who, in the absence of appropriate jobs at home, are forced to seek employment overseas.

The pursuit of science, as a career leading to technical expertise or as a research discipline, is invested with little of the prestige attached to the liberal professions. University teaching is hampered by the national political instability which, combined with the volatility of the student population, can result in the suspension of classes at any time for periods of several months. The majority of teachers have little more than a basic degree or may even be unqualified, having obtained their position through personal influence. Even those with full-time positions often work at a second job, leaving their classes to one of their assistants, usually a final year student. In science, practical training is limited as there are often no laboratory facilities, or materials are restricted to what the student can provide from his own funds. Postgraduate education does not exist at present, but even if the plans for it were implemented, who would be the teachers? In short, the few students who wish to become scientists have little choice but to seek their training overseas.

Upon their return they may find limited opportunity for research except in the fields of cosmic ray physics or

Drawings by Professor P. J. Schofield of the University of New South Wales, Australia

high altitude physiology. Bolivia's geographical position, with a range of altitudes up to 7,000 metres above sea level and a large proportion of the population living at around 4,000 metres, provides an ideal laboratory for this type of research and has attracted investment from overseas in establishing research facilities in these areas.

Yet even here an overseas-trained scientist does not always find himself in a hospitable environment. His newly won diplomas may not be recognised, he may find difficulty in getting on to the university payroll and he will inevitably encounter pressure from his peers to conform to the sedentary existence of preparing reports or feasibility studies rather than hazard the difficulties of performing a concrete task. While his knowledge of his discipline may be superior to that of his colleagues, his adroitness and experience in the political realm may not match their skills. If this is the case, he may be lost. The few scientists who do succeed in establishing themselves in a productive research career do not always remain there. Summary dismissal for political reasons may terminate their work, or, by a more insidious process, they may find their efforts spread over too many projects, and their energies dissipated by travel overseas to consult upon the development of their country.

A better climate for science seemed in store at the end of 1977, when President Banzer established by supreme decree a Council of Science and Technology with the powers to

assess and direct scientific research within the country. This was the first time that any Bolivian government had recognised that current research could be of use, and that further investment in research might assist in the development of the country. The council has not, however, received a great deal of financial support, and it may be only a gesture in response to pressure from the Organisation of American States and the United Nations. Nevertheless, despite two changes in the military government and the usual administrative reshuffling that accompanied these, it manages to survive and is now making its plans known.

The scheme of development which is envisaged in the council's early reports is implicitly that of the construction of a Western-styled, capital-based, consumer society. Progress towards this is to be achieved by the further exploitation of mineral resources, the production of cash crops for the export market and the expansion of industry with the aid of foreign technology. The scientist is seen as the source of new technologies which will help relieve the stranglehold imposed by foreign corporations. The council sees the promotion and funding of local research, however, as the responsibility of industry, or of the developed world through the establishment of an international fund. But what investor is going to be so benevolent as to buy his way deliberately out of a market? Institutions such as the International Development Bank, after all, do not invest their money for love.

In keeping with the council's broadly defined goals, steps have been taken through the universities and their associated institutions to support on-going research which has the potential for immediate economic impact, and to encourage specific projects of an applied nature. Such changes are to be made within the framework of the present budgetary restraints, a move which seems to lead inevitably to the demise of cosmic ray physics and high altitude physiology. On the other hand, the futility of supporting too many specific projects is revealed by a glance at any of the laboratories throughout the country, many of which house elaborate equipment that has never been operated or even installed. The situation is invariable: the initial project grant failed to account for the services of a skilled technician, a part was missing or an essential chemical not ordered, and by the time funds were available again and the deficiencies made good, time had passed, enthusiasm waned and the research team disbanded.

The agricultural research station at Patacamaya is one project which has fulfilled its aims. But the agronomists who are bent on emulating an agricultural system designed to suit another physical and economic environment often find it difficult to see past the superstition and ignorance that surrounds them. Even if they attempted to make contact with their neighbours, the chances are that there would not be a common language in which to communicate their ideas. The agro-



Farm tools for sale in La Paz market



"A pervasive air of forlornness . . ."—pictures by John Hutton

nomist may speak only Spanish, the sole idiom in which education is available, while the native farmer may speak only Aymara, the native tongue of the high plateau. Disillusionment is mutual. The frustrated scientist may well question the validity of sophisticated techniques such as ova transplantation or investigation into animal genetics when there seems to be little scope for the implementation of this kind of technology. No wonder that his sharper colleagues have taken office jobs in La Paz or that almost half the class with whom he graduated have left Bolivia permanently.

The experience of other Latin American countries suggests that investment into applied research only is in fact a costly and inefficient path to the development of new technologies. It is important for these countries to



encourage basic research, develop teaching standards and invest in library facilities: in sum, to construct the base of an indigenous establishment which could eventually define and resolve scientific problems within the context of the society.

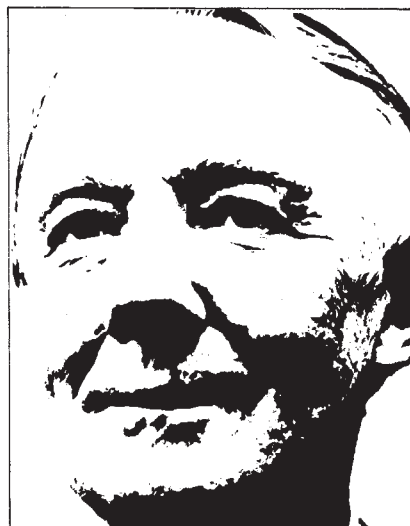
In a country which has seen more than 190 presidents in the 150 years since colonial rule ended, and where government is largely a question of survival, a change of heart is essential if a longer view of investment in scientific research is to be propagated. Meanwhile, Bolivia has few safeguards against the encroachment of modernity and almost no inherent means of curbing the aspirations of a ruthless dictator or foreign investor. Given the present state of the society, it seems that Bolivian science and technology will remain the artefacts of the more developed powers. One wonders, then, how much longer will the agronomist and the native farmer, each surrounded by the trappings of his own culture, continue to gaze uncomprehendingly at each other's flocks across the fence? □

Double standards

TWENTY years ago in Britain there was considerable concern that pesticides might wipe out much of our wildlife. Thousands of corpses of seed eating birds were found in the cornfields of East Anglia, poisoned by aldrin or dieldrin, used as a seed dressing to protect the newly-planted grain from attack by the wheat bulb fly. Hawks which preyed on these seed eaters, and foxes and badgers which ate their corpses, were also affected, and the population of the Peregrine falcon was eliminated from many parts of the country. When the cause of this holocaust was recognised, British farmers and pesticide manufacturers agreed in 1962 to a voluntary ban on the more dangerous use of these pesticides, and since then the situation has greatly improved. The slaughter of seed eating birds has ceased, and the populations of predators have increased considerably. There are still isolated incidents when pesticides kill numbers of birds and other animals, usually arising from the misuse of the chemicals, but in general the situation is satisfactory.

This improvement in Britain occurred as a result of the cooperation of naturalists, scientists and industry. Bodies like the voluntary organisations of ornithologists, the Royal Society for the Protection of Birds and the British Trust for Ornithology, worked closely with the government supported Nature Conservancy and the official Ministry of Agriculture to spell out the recommendations of the Pesticides Safety Protection scheme with the members of the chemical industry. It is perhaps significant that their successful regulations were brought into force some time before the appearance of Rachel Carson's book, *Silent Spring*, which is often thought to have alerted people to the danger of pesticides. Scientists had been well aware of the dangers for several years, and, as I have indicated, had done something practical about them. I fear that the main effect of *Silent Spring* was to make the uninformed over-react, and to deny the safe use of beneficial chemicals to many people throughout the world.

However, the situation varies from country to country. It has often been alleged that while the chemical industry has been very responsible in developed countries like Britain and other parts of Western Europe, where they have restricted their sales of chemicals known to endanger wildlife and the environment, they have behaved quite differently overseas. Up



KENNETH MELLANBY

till now I have seen little evidence that they are dumping chemicals abroad that they cannot sell in western countries. However, recently on the shores of Lake Titicaca, 3,812 m above sea level in the Peruvian Andes, I saw a huge, ugly hoarding advertising aldrin on behalf of the Shell company. Quite apart from the visual pollution of a beautiful previously quite unspoiled landscape, I doubt the wisdom of this commercial enterprise. I also saw, in the offices of the government agricultural officer at Junin, only a few kilometres from the even higher Lake Junin (4,081 m), a pictorial calendar advocating the use of a whole battery of organochlorine pesticides the use of which is strictly controlled or even banned in most developed countries.

I would be the first to agree that medical and dietary problems in developing countries are such that pesticides are essential, and that to save human lives wildlife may sometimes have to be put at risk. With skilled operatives, there may be a place in Peru for aldrin. But all countries, developed and developing, need a code of practice something like that so well observed by industry in Britain. If substantial amounts of aldrin get into the hands of the unsophisticated peasant farmers of the high Andes, the chemical is bound to find its way into the lakes and rivers. There it could well be concentrated in the bodies of the fish and other aquatic life, and could then be passed along the food chain to the unique birdlife of the region. The result could be an ecological disaster. The economic gains to the chemical industry or to the farmers are surely not sufficient to justify such risks.

news and views

Why do messengers wear caps?

from M. J. Clemens

DURING the past decade we have learned a great deal about the structures of messenger RNA molecules in both bacterial and higher cells. Pains-taking work in several laboratories has brought molecular biologists from a position of doubting whether such entities really existed to one of considering which particular structural features enable these molecules to function as the efficient carriers of genetic information in living cells. One of the most exciting (and unexpected) developments in this story was the discovery of the 'cap' structures at the 5' ends of most eukaryotic (but not bacterial) mRNAs. These caps comprise a methylated guanosine residue linked by a 5'-5' triphosphate group to the first coded nucleotide of the RNA; the latter, and the subsequent nucleotide in the sequence, can also be methylated, giving rise to structures of the type $m^7G(5')ppp(5')X^m pY^m$.

There is now a considerable amount of information concerning the biosynthesis and the functions of cap structures in eukaryotic cell and viral mRNAs (reviewed by Shatkin *Cell* **9**, 645; 1976 and more recently by Filipowicz *FEBS Lett.* **96**, 1; 1978). Several possible roles for caps have been proposed, including stabilisation of mRNAs against degradation by 5'-exoribonucleases. Greatest attention, however, has been paid to the influence of the structures on the efficiencies of translation of mRNAs. Several complementary studies, which have included the use of uncapped and capped molecules as templates for cell-free protein synthesis, and investigation of the effects of compounds which resemble the cap structures, have revealed that the presence of a cap usually enhances the ability of a messenger to code for a polypeptide chain. In the cases where the mechanism of this enhancement has been investigated, it appears that capped mRNAs bind to ribosomes more rapidly and to a greater extent than their uncapped counterparts.

This important observation has raised the additional question of how

those mRNAs that are not normally capped function as templates for protein synthesis; in particular, how do bacterial messengers cope with this problem and what would be the effects of introducing caps on to these molecules? Some of the answers to these questions are supplied by two papers by Paterson and Rosenberg in this issue of *Nature* (page 692). They have found that several prokaryotic mRNAs can be translated quite efficiently in a cell-free system derived from wheat germ provided that the 5' ends of these messengers are modified by addition of a methylated cap structure. This modification was achieved in two ways, either using the endogenous cap-forming activity of the wheat germ system with S-adenosyl methionine as methyl donor, or using enzymes purified from vaccinia virus to add the cap and then re-extracting the RNA before adding it to the cell-free system. By such manipulations the efficiency of translation of defined mRNAs of bacteriophage λ and of the *E. coli gal* mRNA could be made comparable with that of the eukaryotic globin mRNA.

These results must indicate considerable evolutionary conservation of the mechanism by which the protein synthetic machinery recognises its templates; apart from the absence of a cap the normal structural features of bacterial mRNAs appear to be sufficient to permit their utilisation by the wheat-germ extract. But how do bacterial ribosomes bind to uncapped bacterial mRNAs? Clearly there must be different structural features of the latter which facilitate such interactions. One such feature is the presence of nucleotides on the 5' side of the initiating AUG codon which can form base pairs with sequences at the 3' end of 16S ribosomal RNA (Shine and Dalgarno *Proc. natn. Acad. Sci. U.S.A.* **71**, 1342; 1974). It is interesting that eukaryotic mRNAs, in contrast, generally cannot form extensively base-paired complexes with eukaryotic 18S rRNA and, as pointed out by Rosenberg and Paterson, neither can

the mRNAs they have used. One reason for the appearance of cap structures in higher cells may therefore be to replace the direct mRNA-rRNA interaction which facilitates ribosome binding in prokaryotic organisms. Because cap structures appear not to bind to ribosomes directly, but require one or more additional protein initiation factors for this process, we may also have a partial explanation for the greater number of such factors needed for successful eukaryotic protein synthesis, relative to the prokaryotic process.

We are now in a position to see in outline how eukaryotic cell ribosomes may find the AUG initiation codons on mRNAs, according to the model recently proposed by Kozak (*Cell* **15**, 1109; 1978). Native small ribosomal subunits carrying initiator methionyl-tRNA bind at or near the 5' cap structure and then move along the RNA, scanning its sequence until an AUG is soon encountered, which will be complementary to the anti-codon on the tRNA. The large ribosomal subunit then attaches and protein synthesis can commence. Thus, polypeptide assembly begins at the first AUG codon occurring at the 5' end of the RNA. A prediction from this model is that internal AUG triplets are not used as initiation sites and that eukaryotic ribosomes cannot translate cistrons other than the 5'-proximal one in polycistronic mRNAs, even after cap addition. This prediction is borne out by the results described by Rosenberg and Paterson, making it tempting to suggest that cap-dependent initiation is the reason for the absence of functional polycistronic mRNAs from higher cells.

In addition to the mechanistic implications of these experiments, some practical considerations arise concerning the efficient expression of foreign

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genes in both animal and bacterial cells. The addition to prokaryotic mRNAs of cap structures, with or without the non-coding sequences adjacent to them, by means of chemical, enzymatic or genetic engineering

techniques, could amplify the synthesis of certain bacterial gene products in higher cells (assuming the RNA can be introduced successfully into the cells). Conversely, there may not be a need for 5' caps on eukaryotic mRNAs

which are to be translated within bacterial cells. Other structural features of the molecules, including their potential ability to base pair with 16S rRNA and to be recognised by bacterial factors, may be more important. □

Processing of protein precursors

from D. F. Steiner

WORK with a wide variety of secretory proteins has by now amply substantiated the 'signal hypothesis'. Proteins that are eventually going to be secreted are synthesised on ribosomes attached to the endoplasmic reticulum (ER) and transferred into the lumen of the ER from where they are finally released from the cell. The signal hypothesis states that these proteins initially contain an N-terminal 'signal' region which during translation guides the attachment of the ribosomes to the ER. The signal sequence is cleaved off shortly after the protein is segregated into the cisternae of the ER prior to secretion.*

One difficult exception to the signal hypothesis has been ovalbumin, which is secreted into the ER and glycosylated without the apparent participation of a cleaved signal sequence. However, the laboratories of both R. Palmiter (University of Washington, Seattle) and G. Blobel (*J. Cell. Biol.* **79**, 567; 1978) have found that ovalbumin competes with other presecretory proteins for transfer into microsomes in reconstituted translational systems, implying the presence of a functional leader sequence. This apparent paradox has now been resolved by a dramatic announcement by V. Lingappa *et al.* from Blobel's group (Rockefeller University) that a prepeptide-like 'signal equivalent' sequence can be released from ovalbumin by tryptic digestion and that this sequence is located more than 200 residues into the molecule. This region contains all the competing activity, and from its position and composition can be inferred to contain a modified version of the typical N-terminal presequences of several other oviduct presecretory proteins (studied by Palmiter's group). It is not yet known whether this region functions as a signal peptide in the transfer of ovalbumin across the ER membrane,

and if it does, how the very long N-terminal region of the nascent chain could be transferred.

However, the finding of an apparent signal sequence at an internal site rather than at the N-terminal end itself, lends support to the 'loop' models of protein transfer into the ER, as against the membrane pore model, originally put forward by Blobel and Dobberstein, in which it was postulated that proteins are threaded through membranes, N-termini first. The loop models proposed by Inouye (*A. Rev. Biochem.* **47**, 481; 1978) and by Steiner *et al.* (*Proc. int. Symp. Proinsulin, Insulin and C-peptide*, 1978, Elsevier, in the press) suggest that the lipophilic signal sequence interacts with the membrane and assumes an inverted transmembrane orientation. Steiner and colleagues at the meeting suggested that the 9-10-residue region of the signal sequences interacts with components spanning the membrane and that successive formation of hydrogen bonds within the apolar membrane core between the pre-existing and the entering prepeptide strands produces a peptide loop across the membrane. Such a receptor mechanism could depend more on secondary than on primary structure and could explain the apparent lack of specificity in the binding of the various presequences. The loop mechanism is supported by preliminary evidence from P. Quinn *et al.* (University of Chicago) that the N-terminal region of the lead peptides of rat growth hormone and human placental lactogen (HPL) remain outside vesicles in *in vitro* transfer experiments and are released into the medium as small residual peptides.

As G. Scheele (Rockefeller University) pointed out in discussion, the loop model may provide a mechanism for incorporating multiply inserted proteins into membranes. It also seems possible that it might provide a means for inserting transmembrane proteins with 'inverted' N → C polarity across the membrane.

Converting enzymes

Protein processing is not confined to the cleavage of signal sequences, however. Most polypeptide hormones, for example, are synthesised as much longer 'prohormones' which are subsequently processed to produce the biologically active product. The nature of the trypsin-like and carboxypeptidase B-like enzymes responsible for the cleavage of the paired basic residues (which mark the cleavage sites) in many of the prohormones remains unclear. The cleavage of nerve growth factor and epidermal growth factor (discussed by E. Shooter, Stanford University) is carried out by specific trypsin-like peptidases which form one of the subunits of the high molecular weight complexes in which these growth factors occur. The complexes thus seem to resemble proteinase/inhibitor complexes.

The signal peptidase activity in detergent-treated microsomes has been partially characterised. There is now general agreement that it is a neutral endopeptidase latent within untreated microsomes and is probably associated with the luminal face of the microsomal membrane. Its inhibition by high concentrations of 1,10-phenanthroline and several thermolysin inhibitors (M. Zimmerman *et al.* Merck, Sharpe and Dohme, Rahway, New Jersey) suggest that it may be a metalloprotease.

An interesting viral cleavage system is that of Sindbis virus. In this case a multicistronic mRNA encodes the capsid protein followed, without punctuation, by the two virus glycoproteins which become membrane-associated. Schlesinger *et al.* (Washington University, St Louis) have shown that the nascent polypeptide itself cleaves off the N-terminal capsid portion autocatalytically before tandem translation of the glycoproteins which are then vectorially coupled to fatty acids within the membrane and cleaved

*A conference on Precursor Processing in the Biosynthesis of Proteins was held in New York City on 2-4 May, sponsored by the New York Academy of Sciences. It was organised by D. F. Steiner and M. Zimmerman.

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to their mature peptide forms. The tandem arrangement of these proteins suggests again the possibility that a functional signal sequence need not be at the N-terminus of a nascent polypeptide.

Genetics

The advent of recombinant DNA techniques has greatly facilitated studies of the structure of the genes coding for protein precursors. Most remarkable at the meeting was the wealth of information gained from structural studies at the nucleotide level by Howard Goodman and coworkers on cloned mRNAs for rat and human growth hormones and the related peptide human chorionic sommatomammotropin (HCS). Studies of the corresponding chromosomal genes of HGH and HCS indicate the presence of multiple intervening sequences, while the gene for rat insulin I from the hooded rat was found to contain only a short intervening sequence in the 5' untranslated portion of the gene. Further studies on cloned rat preproinsulin genes derived from a Wistar-related strain, reported by P. Lomedico *et al.* from W. Gilbert's laboratory (Harvard University) confirmed the presence of a single intron (of 119 bp) upstream from the initiation codon in the gene for rat preproinsulin I, while the gene coding for preproinsulin II contained an additional intron of about 500 nucleotides between the region encoding amino acids 38 and 39 of the proinsulin II peptide chain (thus being in the C-peptide region). Their finding of nuclear RNA molecules larger than preproinsulin mRNA, confirming an earlier report by Duguid *et al.* (*Proc. natn. Acad. Sci. U.S.A.* **75**, 3249; 1978) indicates that the intervening sequences are transcribed into precursor mRNAs. Similarly in studies on growth hormone biosynthesis in rat pituitary GH cells pulse-labelled with uridine F. C. Bancroft *et al.* (Sloan-Kettering Institute, New York) have identified a nuclear 20S mRNA precursor which is slowly processed to the mature 12S mRNA of the hormone.

N-terminal partial sequence studies of the pro-opiocortin precursor of the cell-free translation product of its mRNA by E. Herbert *et al.* (University of Oregon, Eugene), and by N. G. Seideh *et al.* from M. Chretien's laboratory (McGill University), have confirmed the presence of a signal sequence predicted from the recently completed nucleotide sequence of the DNA (Nakinishi *et al.* *Nature* **278**, 423; 1979). They have also established the presence of a cleavage site between Gly-Trp, 26 residues in from the initiator codon. R. Mains and B. Eipper (University of Colorado, Denver) presented recent studies showing that one of the pro-opiocortin precursors (31K) is processed

differently in pars intermedia than in the anterior pituitary, favouring production of α melanocyte stimulating hormone in the former and of adrenocorticotrophic hormone in the latter tissue. The basis for this difference in processing in the two tissues is unknown.

An exciting new approach to studies at the nucleotide level of less abundant mRNA species came from the demonstration by K. Agarwal *et al.* (University of Chicago) that synthetic nucleotide primers can be utilised to detect and selectively transcribe into cDNA those mRNAs which are present below the 1% level in poly A-containing RNA fractions from tissues. Application of this approach to studies on gastrin mRNA mixtures led to the successful identification of a 620-nucleotide mRNA coding for gastrin G34 and indicated the presence of additional amino acid sequences on both sides of the G34 coding region (*Proc. natn. Acad. Sci. U.S.A.* **76**, 1770; 1979). These results predict that progastrin is 110–140 amino acids long and provide the necessary means to purify and ultimately clone the mRNA for this hitherto unexplored precursor molecule. This method is obviously of wider applicability and will undoubtedly provide a new strategy for identifying and amplifying important gene and mRNA structures. □

Is unification really true?

from Norman Dombey

THERE is a general consensus in the world of elementary particle physics that electromagnetic interactions and weak interactions (responsible for nuclear β -decay) are just different aspects of the same theory (*News and Views* **278**, 209; 1979). The predictions of the standard unified gauge theory of weak and electromagnetic interactions based on the group $SU(2) \times U(1)$ have been verified in every high energy scattering experiment so far performed, culminating in the remarkable demonstration of parity violation at Stanford last year when a difference was observed in the scattering of left-handed electrons and right-handed electrons off a deuterium target (*News and Views* **274**, 11; 1978). Indeed the July/August issue of the *CERN Courier* then remarked that the only remaining problem in the field was to find a suitable name for the unified theory.

A note of caution was sounded recently by Barut (*Nature* **278**, 692; 1979) who noted that the crucial test for a unified theory was the observation of the spectrum of gauge particles or quanta of the theory

with masses of the scale $e/G^{\frac{1}{2}} \sim 90$ GeV where e is the electric charge and G is the Fermi constant of the weak interactions. The combination of the two basic coupling constants in this way would be the decisive feature of a unified theory.

I shall attempt here to take this comment further and to review critically the present evidence for unification. I will indicate in fact that the successes of the standard theory could be the result of the inclusion in that theory of assumptions which have nothing to do with the unification of weak and electromagnetic interactions.

The experiment situation as far as high energy scattering is concerned can be summarised thus: all neutrino neutral current experiments with nucleon targets whether inclusive (that is of the form $\nu N \rightarrow \nu X$ where X is unobserved) or exclusive (for example, $\nu N \rightarrow \nu N$, $\nu N \rightarrow \nu N \pi$) together with the Stanford polarised electron experiment are consistent with a neutral weak current of the form written down by Glashow (*Nucl. Phys.* **22**, 579) in 1961 in his pioneering attempt to unify weak and electromagnetic interactions through the group $SU(2) \times U(1)$. This current is

$$J_\mu^W = V_\mu^3 + A_\mu^3 - 2\sin^2\theta J_\mu^{\text{em}} \quad (1)$$

where V_μ^3 and A_μ^3 are the neutral isospin components of the vector and axial currents whose charged components constitute the current of the normal $V-A$ theory of β -decay, and J_μ^{em} is the electromagnetic current. Experimentally $\sin^2\theta = 0.24 \pm 0.02$ (*Proceedings of Neutrino 78*, 253, Purdue University Press, 1979).

Glashow, however, was unable to predict the strength of neutral current scattering by neutrinos compared with charged current scattering (that is $\nu(\bar{\nu}) + N \rightarrow \mu^-(\mu^+) + X$) because he did not know the relation between the mass M_Z of the neutral gauge particle Z^0 of his theory compared with the mass M_W of the charged gauge particles $W^\pm$. The strength of charged current scattering is given by the Fermi constant G where $G/\sqrt{2} = g^2/8M_W^2$ as a W^+ or W^- is exchanged in the process ($g = e\sin\theta$ is the weak coupling constant). For scattering by neutral currents the appropriate Fermi constant G' is given in $SU(2) \times U(1)$ by $G'/\sqrt{2} = g^2/16M_Z^2\cos^2\theta$ as now a Z^0 is exchanged.

This problem was solved by Weinberg (*Phys. Rev. Lett.* **19**, 1264) in 1967 by using the Higgs mechanism of spontaneous symmetry breaking to generate the masses M_W , M_Z thereby finding that $M_W = M_Z\cos\theta$. Hence the relation between G' and G in the standard theory is just

$$G' = \frac{1}{2}G \quad (2)$$

The best value for the neutral current

strength in neutrino scattering is conveniently written in terms of $\kappa = M_Z \cos \theta / M_W$. Then $\kappa^2 = 0.98 \pm 0.05$ (*Proc. Neutrino 78*, 253) in close agreement with the Weinberg value $\kappa = 1$.

Thus the standard theory predicts with remarkable accuracy both the form and the strength of the weak neutral current in high energy scattering experiments. The only difficulty at present in the experimental verification of the theory lies in the conflicting results on atomic parity violation in bismuth (*Proc. Neutrino 78*, 417; *News and Views* 264, 505; 1976) and thallium (*Phys. Rev. Lett.* 42, 343; 1979): the Washington experiment which has the smallest reported errors disagrees by some seven standard deviations from the predictions of the standard theory. These experiments, however, are very difficult and require for their interpretation a model of heavy atoms of uncertain validity.

Hence the general consensus that the standard theory has been verified. The sceptic must now remark that all the data so far observed are consistent with the old Fermi four-fermion theory of weak interactions, provided that neutral currents are included with the form (1) and strength (2). No additional structure in weak interactions due to the existence of gauge particles $W^\pm$, Z^0 with their prescribed masses of 78 and 90 GeV respectively has been observed, nor is there any experimental evidence yet for the scalar Higgs particle required by the theory. Neither (1) or (2) is especially complicated so there may well be a simple explanation of these two relations which does not require the untested features of the standard unified theory: in particular the predicted masses for $W^\pm$ and Z^0 and the existence of the Higgs scalar.

Hung and Sakurai (*Nucl. Phys.* B143, 81; 1978) did indeed find recently an extremely simple explanation of (1) and (2). They showed that starting with the isospin- or SU(2)-conserving V—A theory of weak interactions including neutral currents due to Bludman (*Nuovo Cimento* 9, 433) in 1958, then the W^0 which couples to the neutral weak current and the photon γ which couples to the electric current must mix quantum mechanically. This is because both electrons and quarks can be left-handed (for the V—A weak coupling) and charged. If this calculation is done carefully, making sure that the photon is massless both before and after mixing, then not only is the Glashow current (1) obtained with $\sin^2 \theta$ as a measure of the mixing—which it is in the standard theory anyway—but also the mixing does not change the relation $G' = \frac{1}{2}G$ which comes simply from isospin invariance in

the underlying theory. Thus the two successful predictions of the standard theory are obtained from a mixing calculation with no extra assumptions about unification, gauge theories or Higgs particles. From this point of view all the successes of the standard unified gauge theory are just due to the correct incorporation of γ — W^0 mixing in the formalism.

Hung and Sakurai consider that their calculation is an alternative approach to weak and electromagnetic interactions that contains the standard theory and may but does not necessarily, give different results. They emphasise that from the mixing point of view, $\sin^2 \theta$ should be

considered as a function of momentum transfer q and not as a fundamental constant, and that the electron and muon neutrinos would have different interactions with the electromagnetic field, and therefore different values of $\sin^2 \theta$ should be associated with different neutrinos. Their motivation seems to be mainly a dislike of the Higgs particle which is a necessary ingredient of the standard theory but whose mass is arbitrary and is not predicted by the theory. The number of Higgs particles is also arbitrary but in the standard theory, the simplest choice of one Higgs is made, thereby obtaining relation (2).

Although Hung and Sakurai do not



A hundred years ago

SOCIETIES AND ACADEMIES

BOSTON (U.S.A.)

American Academy of Arts and Sciences, May 14.—Hon. Charles Francis Adams in the chair.—Dr. H. P. Bowditch presented a new form of plethysmograph differing from those of Mosso and van Basch in the method adopted for securing a constant level of the fluid in the receptacle connected with the apparatus which contained the body whose changing volume was to be measured. The method consisted in suspending the receptacle (a large sized test tube) to a delicate spiral steel spring of which the length and strength were so adjusted that the weight of the fluid flowing into the test tube caused an elongation of the spring precisely equal to the rise of the fluid in the test tube itself. Thus the absolute level of the fluid in the receptacle remained unaltered, and a constant pressure was maintained upon the surface of the organ to be measured. An index attached to the lower end of the spring recorded upon a revolving cylinder covered with smoked paper the flow of the fluid into and out of the receptacle.—Mr. N. D. C. Hodges gave two new proofs of the dimensions of molecules, one based upon the properties of water and aqueous vapour, the other upon superficial tension and considerations of the depth of the superficial layer of molecules upon sheets of platinum.—Prof. Pickering exhibited a new form of photometer for measuring the light of a nebula or comet, by comparison with a star thrown out of focus. The method employed eliminated the effects of moonlight or twilight. He also proposed to denote the light of these bodies in stellar magnitudes. Thus a portion of a nebula would be of the twelfth magnitude, if of the same brightness as a twelfth-magnitude star spread over a circle one minute in diameter.

Evolution Old and New

MR. A. R. WALLACE writes, in *NATURE*, vol. xx. p. 143, that, according to the theory which I support, Australian (and more especially Queensland) sheep should show a tendency to grow a scantier and thinner fleece than their English ancestors. "If Mr. Butler," he continues, "could adduce on good authority such a fact as this, he would have some evidence in his favour, instead of which he can only make suppositions."

I never was in Australia, but had some years' experience of sheep-farming in New Zealand. It was generally believed, in my time, that fleeces soon became short and hairy in Queensland, and even in the more northern part of New South Wales. You must, however, have many readers who could tell us what the facts are. May I hope that you will kindly insert this, so as to get the matter settled by eliciting information from a competent authority? I am speaking, of course, of sheep that are left to the effect of the climate, without being frequently crossed with rams from colder countries. Do the fleeces of such sheep deteriorate in Queensland? S. BUTLER

June 12

Oxygenated Rain

ON Thursday, June 12, at half-past eleven in the morning, a remarkable shower of rain fell over London, which might almost be described as "effervescing;" the drops whilst falling appeared to be colourless and perfectly transparent, but on striking against any solid surface they became milky, and on close examination it was evident that this cloudy appearance was caused by a number of very minute air-bubbles, which rapidly increased in size, and then burst. From the bleaching power which this rain appeared to have, I am led to believe that there was nascent oxygen in the gas thus evolved. Those who traverse the streets of London in the early morning may now and then observe the red colour of all bright iron-work in the pavement, such as coal-plates, &c., due to the oxidising influence of a thunder-shower in the night; this effect does not follow every thunder-shower, but seems to indicate a peculiar atmospheric condition. Have any memoranda on this subject been recorded?

EDWARD SOLLY
From *Nature* 20, 19 June, 169, 188; 1879.

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claim that a theory different to the standard theory results from their work, it is possible to envisage a radical alternative to the standard theory starting from γ - W^0 mixing. The motivation now is that if $\sin^2\theta$ were essentially a radiative correction as these ideas suggest then $\sin^2\theta \approx \alpha \approx 1/137$ whereas experimentally $\sin^2\theta \approx 1/4$. This means that the radiative corrections must be much larger than expected; yet they can only be much larger if higher order weak interactions are important. This implies that $g^2/4\pi \gtrsim 1$ whereas in the unified theory $g = e\sin\theta$ so $g^2/4\pi < \alpha$. Hence now $M_W, M_Z \gtrsim 1/G^{\frac{1}{2}} = 300$ GeV.

Of course the alternative approach is not a complete theory, it is just a calculation of quantum mixing which must be part of any theory. So if the radical alternative is taken seriously, it is necessary at some stage to consider what happens at high energies ($\gtrsim 300$ GeV) where the four-fermion theory must break down. What does happen cannot yet be predicted but significant progress has been made recently on treating the four-fermion interaction as a serious theory in its own right by Cooper, Guralnik and their collaborators (*Ann. Phys.* **109**, 165; 1977; *Phys. Rev. Lett.* **40**, 1620; 1978; *Phys. Rev. D* **19**, 549, 562; 1979). The ideas here are based on the theory of superconductivity which of course also involves a four-fermion interaction, albeit one which is non-relativistic. The calculations are not performed perturbatively but in a mean field approximation as in many-body theory. Both vector (gauge) and scalar (Higgs) particles now appear as dynamical excitations of the theory, not as elementary entities, thereby removing the arbitrariness of the Higgs particle mass which many see as the most serious failing of the standard theory.

The theory of superconductivity may well provide a good analogy for weak interaction theory. Englert (International Centre for Theoretical Physics, Trieste, Report IC/76/92; 1976) has pointed out that the Higgs phenomenon has already been discovered experimentally, not in particle physics but in the Meissner effect. The range of penetration of the magnetic field is given by the photon mass induced by spontaneous symmetry breaking of the Ginsburg-Landau Lagrangian through the Higgs mechanism. Here the Higgs scalar can easily be understood: it is just the bound state Cooper pairing of the electrons of the BCS theory. The Ginsburg-Landau approach to superconductivity in terms of vector and scalar order parameters is the phenomenological theory, whereas the basic physics lies in the BCS four-fermion theory. In the same spirit, the standard unified theory could be regarded as a phenomenological theory of weak interactions valid in the region $q^2 \ll M_W^2$, or perhaps, $m_e^2 \ll q^2 \ll M_W^2$ (m_e is the electron mass), while the four-fermion theory is funda-

mental. In a Los Alamos Report (LA-UR-79-306; 1979) I have noted that the latter possibility would allow $\sin^2\theta \approx 0$ in atomic physics where $q^2 \lesssim m_e^2$ thereby giving a value for atomic parity violation in bismuth or thallium about three times smaller than in the standard theory, a result which agrees with all the experiments performed to within 2.5 standard deviations.

If the radical alternative is correct, then two final remarks are appropriate. The first is theoretical. Weak interactions now become strong at energies of 300 GeV, they do not level off at a typical electromagnetic strength at 90 GeV or so. This provides a physical reason for a grand unification of leptons and hadrons at high energies which is missing from the usual viewpoint. The second remark is experimental. No Z^0 will be seen at LEP for 70 GeV electrons on 70 GeV positrons. To ensure seeing some effects of weak interaction structure a centre of mass energy of at least 300 GeV is needed. $\square$

Oscillations in cellular reactions

by Eleanor Lawrence

A RECENT meeting* in the Black Forest brought together some of those working in the field of cellular oscillating reactions. The periodic rise and fall in concentrations of metabolites or in membrane potential is found in a wide variety of cells. In some cases, such as the membrane potential changes in cardiac pacemaker cells, the physiological significance of the oscillating reaction is clear; in others the oscillatory phenomena may be a consequence of the underlying physico-chemical properties of the components of a particular reaction but may have no further physiological role. However, the general point was made at the meeting that an oscillating reaction provides a stable and energy-efficient signal whose modulation by metabolites, hormones or synaptic input could convey information rapidly and precisely to the cell itself or to its neighbours.

Some order, however arbitrary it

may eventually appear, can be imposed on this collection of diverse and seemingly unrelated phenomena by a division into 'cytoplasmic' oscillators on the one hand, of which the glycolytic oscillator is a prime example, and 'membrane' oscillators on the other. Self-contained models for oscillating membrane potential based on a cycle of inward and outward ionic currents are consistent with much of the experimental evidence. But whether membrane oscillators are in practice completely self-contained or whether they are in some cases driven by underlying internal oscillators is still an open question, and various possibilities were canvassed at the meeting.

Glycolytic oscillator

Undoubtedly the cellular oscillator best characterised at the molecular level is the so-called glycolytic oscillator described by B. Hess (Max-Planck-Institut für Ernährungsphysiologie, Dortmund). Periodic regular oscillations in the concentrations of glycolytic intermediates were discovered first in yeast cells and later in extracts of skeletal muscle and heart. The key component producing the oscillations in this pathway is the allosteric enzyme phosphofructokinase (PFK), which catalyses the phosphorylation of fructose-6-phosphate into fructose-1,6-diphosphate using ATP as an energy source. The oscillations reflect the periodic changes in the activity of PFK (within a certain range of substrate input) in response to its activators F6P, ADP and AMP, and to its allosteric inhibitor ATP, which produces regular pulses of product to which the rest of the pathway is geared. The stability of the oscillations to the stochastic substrate input, expected normally, has been shown by experiments in which input was varied at random. In these conditions the reaction still oscillated at its 'normal' periodicity. Such oscillating reactions have the fascinating property of generating structure in initially homogeneous solutions, as Hess illustrated. When the reaction in a shallow unstirred yeast extract is followed by ultraviolet photography, which picks up the fluctuations in oxidised and reduced pyrimidine nucleotides, the solution soon takes on a regularly pulsing structure composed of discrete domains.

So far, however, evidence that the glycolytic oscillator could provide a basis for other oscillatory phenomena is sparse. R. Chaplain (University of Mainz) is accumulating evidence suggesting a link between glycolysis and the membrane potential oscillations represented by the periodic autonomous firing of certain neurosecretory neurones in the abdominal ganglion of the sea-hare *Aplysia*. I. Levitan and his

*A meeting on 'Cellular Oscillators', organised by M. J. Berridge and P. E. Rapp, and sponsored by the Company of Biologists and Dr Karl Thomae GmbH, was held at Titisee on 22-24 March 1979. The proceedings will be published as Volume 81 of the *Journal of Experimental Biology* due out in August 1979.

colleagues (Friedrich-Miescher-Institut, Basel) with a different neurone in the same ganglion, find that oscillatory behaviour can be profoundly changed by changes in the internal concentrations of cyclic nucleotides. The inhibitory effects of synaptic stimulation can be mimicked by increases in cyclic AMP alone. The enhanced activity induced by vasopressin and oxytocin is simulated by increases in cyclic GMP and cyclic AMP together. These hormones naturally cause a rise in cyclic nucleotide levels. Levitan has also identified hormonal activity resembling that of vasopressin and oxytocin in peptide-containing extracts of molluscan ganglia.

Membrane oscillators

Detailed dissection of the ionic mechanisms of membrane potential oscillations reveals a slightly different picture in different tissues. However, in all cases the oscillatory behaviour appears to be due primarily to a slow outward K^+ current which is normally responsible for membrane hyperpolarisation. As this current gradually inactivates it initiates membrane depolarisation leading into the upswing of the next action potential.

In cardiac pacemakers (the frog sinus venosus and the mammalian sinoatrial node) described by H. Brown and S. Noble (University of Oxford) and the Purkinje fibres described by R. Tsien (Yale University School of Medicine) the dominant pacemaking oscillation in membrane potential can be explained by a self-contained cycle of inward and outward currents which are activated and inactivated by the changes in voltage they produce. Brown and Noble together with D. DiFrancesco have been looking at the mechanism of the inhibition of pacemaking in the heart by acetylcholine and its acceleration by adrenaline. They find that acetylcholine in both mammals and amphibia greatly increases outward potassium current, possibly by opening up a special ACh-activated potassium channel. Adrenaline on the other hand, increases the slow inward Na^+/Ca^{2+} current and also affects a newly discovered additional pacemaking current, overriding the adrenaline-induced increase in outward current.

Although the dominant pacemaking membrane oscillations can be explained by a self-contained membrane model, as Tsien pointed out, pacemaker cells also probably contain a separate 'internal' oscillator largely independent of the main membrane oscillator, but which reveals itself in oscillatory changes in membrane conductance and cell contractility even when the mem-

brane potential is fixed. This internal oscillator is enhanced in conditions that raise internal free calcium, leading Tsien to suggest that the membrane conductance oscillations are driven by a cycle of uptake and release of Ca from some intracellular store, probably the sarcoplasmic reticulum. Increased intercellular free calcium is known to increase membrane permeability to K^+ .

A cycle of Ca uptake and release, this time by the endoplasmic reticulum is invoked by P. G. Nelson (National Institutes of Health) to explain the oscillations in membrane potential seen in L cells subjected to mechanical or electrical stimuli. On electron microscopic evidence of specialised structures closely apposed to the membrane, Nelson proposes a model in which Ca-induced changes in membrane permeability to K^+ are coupled to a cycle of Ca uptake and release from the ER. A similar Ca^{2+} cycle might also drive the periodic contractions in the veins of the acellular slime-mould *Physarum* (described by K. E. Wohlfarth-Bottermann, University of Bonn).

The role of calcium in controlling the frequency of bursts in the molluscan burster neurones was emphasised by R. W. Meech (ARC Unit of Invertebrate Chemistry and Physiology, Cambridge). He suggests that the prolonged inward current carried by Na^+ and Ca^{2+} , which is responsible for the depolarising phase of the burst, is also responsible for a rise in intracellular Ca which then triggers the slowly developing outward K^+ current responsible for the hyperpolarising interburst phase.

Link with glycolysis

A link with both glycolysis and cell Ca concentration is proposed by E. K. Matthews (University of Cambridge) for the membrane oscillations elicited in pancreatic islet cells stimulated to release insulin by glucose. The membrane oscillations themselves are again driven by changes in K^+ conductance but in this case Matthews proposes a link with glycolysis. Glucose metabolism leads to a decreased permeability to K^+ and so to depolarisation. The principal ion entering the cell during the bursts of action potentials superimposed on the crests of the membrane oscillation, is thought to be Ca^{2+} , which could also provide negative feedback, increasing K^+ permeability and initiating the hyperpolarising interburst phase.

One organ in which the physiological role of cellular oscillations is clear although their mechanism is less clear, is the intestine. The smooth muscle in the wall of the stomach and intestine displays fairly slow oscillations in membrane potential—the 'slow wave'—which propagates along the intestine

(J. A. Connor, University of Illinois, Urbana). Contraction is initiated when action potentials are generated on the crest of the wave. In the cat at least, pacemaker activity seems to reside in the longitudinal layer of the musculature. Preliminary attempts to identify the primary oscillator point, in Connor's opinion, to rhythmic modulation of current from an electrogenic sodium-potassium pump, although other metabolic processes may be involved.

Chemical signals

Returning to 'metabolic' oscillators, one of the most spectacular is the aggregation of the amoebae of the cellular slime-mould *Dictyostelium* as a result of the propagation and amplification from cell to cell of a chemotactic signal carried by cyclic AMP. G. Gerisch (Biozentrum, Basel) speculated on possible metabolic control pathways leading to the periodic synthesis of cyclic AMP pulses. On the basis of strong evidence of oscillations in cyclic GMP activity, Gerisch has proposed a tentative scheme of feedback control loops in which cyclic AMP binding to the external receptor leads to an immediate rise in cyclic GMP which then activates adenylyl cyclase leading to increased cyclic AMP production. This in turn damps down the receptor-mediated increase in cyclic GMP. Gerisch sees the oscillations in adenylyl cyclase activity (possibly mediated by phosphorylation of the enzyme) as the primary oscillation in the pathway in contrast to other models which have taken the allosteric change in the receptor on binding cyclic AMP as the key component.

M. Israel (CNRS, Gif-sur-Yvette) described oscillations in the intracellular concentrations of ACh in *Torpedo* electroplax during stimulation. On the apparent fairly long-period rise and fall in intracellular ACh are imposed very short-period changes in ACh concentration which oscillate in phase with ATP concentration. As the amplitude of electrical discharge (reflecting transmitter release) does not oscillate, they conclude that transmitter output is not directly related to ACh concentration.

No unification

Summing up, M. Berridge (ARC Unit of Invertebrate Chemistry and Physiology, Cambridge) pointed the moral of the meeting—that there is no universal oscillator underlying the different phenomena described. Although the influence of calcium pervades many discussions of oscillatory mechanisms and Rapp and Berridge (*J. theor. Biol.* **66**, 497; 1977) have proposed the attractive notion that control loops involving calcium and cyclic nucleotides may be involved in many oscillating reactions, the existence of such controls remains to be demonstrated experimentally, and they are unlikely to be universal. □

review article

Coated pits, coated vesicles, and receptor-mediated endocytosis

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Proteins and peptides can enter cells by receptor-mediated endocytosis, a coupled process by which selected extracellular proteins or peptides are first bound to specific cell surface receptors and then rapidly internalised by the cell. Internalisation follows clustering of the receptors in specialised regions of the cell surface called coated pits that invaginate to form intracellular coated vesicles. It is now recognised that receptor-mediated endocytosis has a fundamental role in the growth, nutrition and differentiation of animal cells.

CERTAIN proteins that bind to receptors on the surfaces of animal cells are rapidly internalised by the cells before they can dissociate from their receptors. This process, which is called receptor-mediated endocytosis, has recently become recognised as an important and general mechanism by which animal cells take up nutritional and regulatory proteins from extracellular fluid. Biologically important molecules known to be taken up by this mechanism include plasma transport proteins (such as low density lipoprotein and transcobalamin II), certain polypeptide hormones (such as insulin and epidermal growth factor), asialoglycoproteins, and lysosomal enzymes. In many cases, rapid internalisation of receptor-bound proteins is achieved by the clustering of receptors in specialised regions of the surface membrane called coated pits that invaginate rapidly into the cell during endocytosis to form coated vesicles^{1,2}. Coated pits and coated vesicles have recently acquired the status of subcellular organelles because they can be separated from other cellular components and because they possess a characteristic structure and protein composition^{3,4}.

We review here some of the experiments that have delineated the process of receptor-mediated endocytosis. We rely heavily on data gathered from extensive study of the low density lipoprotein (LDL) receptor system, which functions to transport cholesterol from plasma into cells^{5,6}. We then review 12 other protein transport systems for which receptor-endocytosis has been documented. From such data, we deduce certain general features of the process that may apply in most types of animal cells.

Receptor-mediated endocytosis as a mechanism for cellular protein uptake

Endocytosis is a general term that refers to the process by which cells ingest extracellular materials by trapping them within inward foldings of the plasma membrane that pinch off from the surface to form intracellular vesicles⁷. Endocytosis occurs widely in protozoa where it has an obvious nutritional role. In

multicellular organisms endocytosis was first identified in macrophages and other phagocytic cells that ingest foreign particulate materials and deliver them to lysosomes for degradation. With the advent of the electron microscope, it was recognised that endocytosis is not limited to phagocytic cells but occurs in all animal cells⁸. The rate of endocytosis in non-phagocytic cells can be appreciable. For example, mouse L cell fibroblasts internalise an amount of cell surface membrane equal to 50% of the cell surface every hour⁷. One reason for such rapid endocytosis now seems clear: it provides a mechanism by which cells take up necessary macromolecules from extracellular fluid in controlled conditions.

Within the past few years, at least 13 systems have been described in which non-phagocytic cells use endocytosis to internalise proteins that have become bound to surface receptors. These systems share four properties that collectively define receptor-mediated endocytosis: (1) The binding component on the cell surface is a receptor in the strict sense, that is, it is a molecule whose function is to bind an endogenous ligand and thereby achieve a physiological effect. This criterion distinguishes these receptors from other cell surface components that serve as binding sites for opportunistic foreign molecules, such as lectins and toxins, and viruses. (2) Internalisation of the ligand is effectively coupled to binding. Once the protein is bound to the receptor, the half-time for internalisation is less than 10 min⁹⁻¹⁸. (3) In all cases for which ultrastructural data are available, the receptor-bound proteins enter the cell through coated pits, either because the receptors spontaneously localise in these regions or because binding of the ligand induces migration of the receptors to coated pits^{2,19-24}. (4) The internalised proteins are usually delivered to lysosomes where they are degraded completely to amino acids^{2,6,12-18,25,26}; occasionally processing of the internalised protein involves delivery to cellular structures other than lysosomes (see below).

The LDL receptor system illustrates each of the four characteristics of receptor-mediated endocytosis. The similarities and differences between the LDL receptor pathway and other systems of receptor-mediated endocytosis are presented in Table 1 and discussed below.

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Table 1 Systems for receptor-mediated endocytosis of proteins

Protein	Cell type	Internalisation via coated pits and coated vesicles	Fate of internalised protein		Selected refs
			Degraded in lysosomes	Other	
Transport proteins					
LDL	Fibroblasts, smooth muscle cells, endothelial cells, adrenocortical cells, lymphocytes	Yes	Yes; cholesterol retained by cells	—	2, 6, 10, 52, 57
Yolk proteins (phosvitin, lipovitellin)	Oocytes (chicken, mosquito)	Yes	No	Delivered to yolk granules	21, 32
Transcobalamin II	Kidney cells, hepatocytes, fibroblasts	Data not available	Yes; vitamin B ₁₂ retained by cells	—	14, 68, 69
Transferrin	Erythroblasts, reticulocytes	Yes	Data not available	Iron retained by cells	22, 60, 61
Protein hormones					
Epidermal growth factor	Fibroblasts, 3T3 cells	Yes	Yes	—	13, 23, 50
Nerve growth factor	Sympathetic ganglion cells	Data not available	Data not available	Carried in vesicles retro- grade up the axon	62
Insulin	Hepatocytes, hepatoma cells, lymphocytes, adipocytes, 3T3 cells	Data not available	Yes	Also delivered to Golgi apparatus and nuclei	12, 25, 50, 63, 64
Chorionic gonado- trophin	Leydig tumour cells, ovarian luteal cells	Data not available	Yes	—	15, 70
β -Melanotropin	Melanoma cells	Data not available	Data not available	Delivered to Golgi apparatus and melanosomes	71, 72
Other proteins					
Asialoglycoproteins	Hepatocytes	Data not available	Yes	—	16, 17
Lysosomal enzymes	Fibroblasts	Data not available	No	Delivered to lysosomes and Golgi-associated structures; enzymes remain active for many days	65–67
α -2-Macroglobulin	Fibroblasts, macrophages, 3T3 cells	Yes	Yes	—	18, 24, 58
Maternal immunoglobulins (IgG)	Fetal yolk sac, neonatal intestinal epithelial cells	Yes	No	Transferred intact in coated vesicles to basal surface of cells where IgG is discharged into fetal or neonatal circu- lation	21, 47–49

LDL receptor pathway as prototype for receptor-mediated endocytosis

Animal cells acquire cholesterol for synthesis of cell membranes by the receptor-mediated endocytosis of the cholesterol-carrying lipoprotein LDL, a large spherical particle (220 Å in diameter $M_r \sim 3 \times 10^6$) that originates in the liver and circulates in the blood. Each LDL particle contains a nonpolar core composed of approximately 1,500 cholesterol molecules that are esterified to long-chain fatty acids. This cholesteryl ester core is surrounded by a polar coat of phospholipid, unesterified cholesterol, and protein⁶.

The receptor-mediated endocytosis of LDL was originally demonstrated in studies of human fibroblasts in tissue culture and has subsequently been found to operate in a wide variety of animal cells^{5,6}. When cells require cholesterol for membrane synthesis, they synthesise a cell surface receptor that binds the protein component of LDL. Within 10 min of binding to the receptor, the LDL particle is internalised and delivered to lysosomes; in the lysosome the protein component is hydrolysed by proteases to amino acids and the cholesteryl esters are cleaved by an acid lipase to generate unesterified cholesterol. This unesterified cholesterol enters the cytoplasmic compartment where it serves two important functions: utilisation for membrane synthesis and regulation of key enzymes in cholesterol metabolism^{5,6}. This latter action assures cholesterol

homeostasis in the cell as well as in the whole organism. Cytoplasmic cholesterol derived from the lysosomal hydrolysis of LDL also controls the synthesis of receptor molecules. Increased cellular cholesterol decreases the number of receptors, thereby preventing overaccumulation of LDL-cholesterol in the cell⁶.

The distribution of LDL receptors on the surface of fibroblasts has been studied by electron microscopy with the use of LDL coupled to the iron-containing, electron-dense protein ferritin^{2,19,20} or by ¹²⁵I-LDL autoradiography²⁷. Between 50% and 80% of all receptors are clustered over the 2% of the cell surface represented by coated pits (Fig. 1a). The membrane in these regions is indented and coated on its cytoplasmic surface by a bristle-coat. As LDL receptors are localised in coated pits even when the cells are fixed with formaldehyde prior to incubation with the LDL-ferritin, it is believed that receptor clustering occurs before the exposure to LDL¹⁹. Immunofluorescence studies with the light microscope show that the clusters of LDL receptors in human fibroblasts are aligned in linear arrays that appear to overlay actin-containing cellular stress fibres²⁸.

LDL-ferritin bound to coated pits at 4°C is rapidly internalised when the fibroblasts are warmed to 37°C². In this process the coated pits invaginate to form coated endocytic vesicles (Fig. 1a-c). After 5 to 10 min at 37°C, LDL-ferritin is seen in lysosomes (Fig. 1d) as the result of their fusion with the

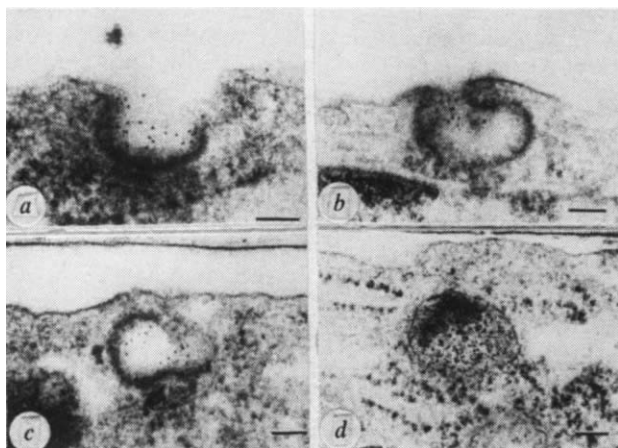


Fig. 1 Electron micrographs showing the sequential binding of LDL-ferritin to coated pits, its internalisation in coated vesicles, and its delivery to lysosomes in human fibroblasts. Monolayers of growing human fibroblasts were incubated with growth medium containing 10% lipoprotein-deficient serum and LDL-ferritin at a concentration corresponding to $47 \mu\text{g ml}^{-1}$ of LDL-protein at 4°C for 2 hours, after which the cells were washed extensively². The cells then received growth medium containing 10% lipoprotein-deficient serum and were warmed to 37°C . After incubation at 37°C for the indicated time, the monolayers were fixed and embedded *in situ* and stained with uranyl acetate and lead citrate². *a*, A coated pit that bound LDL-ferritin (time at 37°C : 1 min). *b*, A coated pit being transformed into a coated vesicle with LDL-ferritin included (time at 37°C : 1 min). *c*, A fully formed coated vesicle that seems to be losing its cytoplasmic coat on the right side (time at 37°C : 2 min). *d*, A lysosome containing LDL-ferritin (time at 37°C : 6 min). Magnifications: *a*, $\times 58,000$; *b*, $\times 50,500$; *c*, $\times 50,000$; *d*, $\times 40,000$. Scale bars, 100 nm.

incoming coated vesicles². The rapid sequence of events visualised in the electron micrographs of Fig. 1 precisely parallels biochemically-derived data on the rapid uptake and degradation of ^{125}I -LDL^{10,11}.

Mutations affecting the LDL receptor

A group of naturally-occurring human mutations has been invaluable in detailing the structure and function of the LDL receptor. Those mutations responsible for the genetic disease called familial hypercholesterolaemia (FH) are most instructive^{5,29}. Three distinct mutations seem to occur in the gene for the LDL receptor^{6,29,30}. The most frequent mutant allele at the LDL receptor locus is designated R^{b0} ; it specifies a nonfunctional receptor. Fibroblasts from patients homozygous for this allele (R^{b0}/R^{b0}) are unable to bind or take up the lipoprotein, even though they have normal coated pits that form normal coated vesicles^{19,30}. A second mutation, R^{b-} , specifies a receptor that is detectable but binds $<10\%$ of the normal amount of LDL. Recently, a rare but extremely informative allele has been identified. This allele, designated $R^{b+,i0}$, specifies a receptor that binds LDL but does not allow the lipoprotein to be internalised^{11,30}. The failure of internalisation is due to the inability of these abnormal receptors to be incorporated into coated pits. Although coated pits of normal appearance are present, the receptors specified by the $R^{b+,i0}$ allele are not inside them. Instead the receptors are scattered over noncoated segments of the cell surface³¹.

The hypothesis that the $R^{b+,i0}$ mutation involves the gene for the LDL receptor implies that the normal receptor must have, in addition to its LDL binding site, a second active site, the internalisation site, which is necessary for the receptor to be recognised as a component of the coated pit. The internalisation site is presumed to be on the cytoplasmic side of the membrane where it can interact with the proteins that constitute the cytoplasmic coat of the coated pit. This formulation implies that

the coat-associated proteins play an active role in the selection of receptors to be incorporated into coated pits and are therefore central to the process of receptor-mediated endocytosis^{30,31}.

The structural role of clathrin

Coated pits and coated vesicles were first described in mosquito oocytes by Roth and Porter, who postulated that these structures mediate the uptake of adsorbed proteins from the extracellular fluid during yolk formation³². Other investigators, including Fawcett³³ and Friend and Farquhar³⁴, found that coated pits and coated vesicles mediate protein uptake in specialised cells of higher animals. Coated pits and vesicles have subsequently been found in virtually all nucleated animal cells.

Coated pits derive their name from their characteristic indented configuration and their fuzzy cytoplasmic coat. Thin section electron microscopy shows that this coat consists of periodically spaced fibres or bristles that radiate from the cytoplasmic surface (Fig. 1*a-c*). Coated pits have been identified in freeze-fracture electron micrographs where they exhibit a larger number of intramembrane particles than the surrounding plasma membrane²⁰. The intramembrane particles in coated pits are approximately 20% larger in diameter than intramembrane particles in non-coated regions of membrane²⁰. These findings suggest that coated pits contain a unique set of transmembrane proteins.

In addition to their role in endocytosis, coated vesicles have been implicated in other types of membrane transport within the cell. They are frequently found associated with the Golgi region where they appear to carry enzymes from the Golgi apparatus to the lysosome^{34,35}. They also play a part in the secretion of proteins by mammary epithelia³⁶ and in the retrieval of excess cell surface membrane in presynaptic neurones³⁷.

Kanaseki and Kadota first analysed the structure of coated vesicles by electron microscopy using negative staining techniques³⁸. They showed that these structures consist of a lipid vesicle surrounded by a basket of protein that is organised in a network of hexagons and pentagons. Pearse developed a rapid method for isolating coated vesicles and made the fundamental observation that the coat of coated vesicles isolated from a variety of animal cells is composed predominantly of a single protein of molecular weight 180,000. She named this protein clathrin^{3,4,39}. Figure 2 shows a stereoscopic view of the proposed structure of the protein network that forms the coat of a coated vesicle.

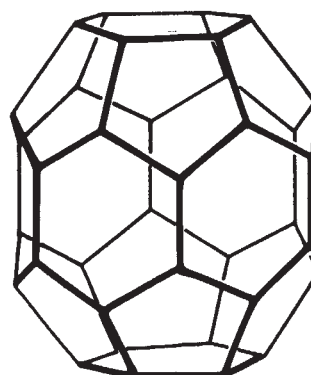


Fig. 2 Proposed structure for the network of protein that forms the coat of a coated vesicle. This structure was deduced by Pearse and co-workers from electron microscopic examination of tilted views of negatively stained coated vesicles isolated from porcine brain^{3,4}. The protein coat, which is organised in a network of 12 pentagons and 8 hexagons, is assembled from 108 clathrin molecules that surround a lipid vesicle. Coated vesicles having other sizes and shapes are believed to be constructed similarly with each vesicle containing 12 pentagons plus a variable number of hexagons. Redrawn from ref. 4 with permission.

Clathrin can be removed from coated vesicles by treatment with urea or protonated amines, indicating that it is non-covalently bound to the surface of the membrane⁴⁰⁻⁴³. With these techniques the protein has now been isolated from brain and chicken oocytes by workers in several laboratories^{3,4,28,39-43}, and it has been shown to have important structural properties that are related to its function. For example, in specified *in vitro* conditions, clathrin will spontaneously aggregate to form a basket-like network of hexagons and pentagons similar to those that surround coated vesicles *in vivo*⁴¹⁻⁴³.

An antibody to the coat protein of bovine brain coated vesicles (directed primarily against clathrin) reacts *in situ* with coated pits and vesicles in human fibroblasts²⁸. The antigen-antibody complex can be visualised by indirect immunofluorescence using light microscopy²⁸ or by the immunoperoxidase technique in the electron microscope (Figs 3 and 4)²⁸. In the electron microscope, the immunoperoxidase-stained clathrin can be shown to underlie coated pits (Fig. 3a), including those that have bound LDL-ferritin (Fig. 4a), as well as coated vesicles (Fig. 3b and c), again including those that have internalised LDL-ferritin (Fig. 4b). Under the light microscope, the coated pits of human fibroblasts show a linear alignment similar to the linear alignment of LDL receptors²⁸. However, in other cells, such as Chinese hamster ovary cells, neither the LDL receptors nor the coated pits show a linear distribution, indicating that a linear arrangement is not essential for coated pit function.

A working model for clustering of LDL receptors in coated pits of human fibroblasts

The genetic, biochemical, and morphological data obtained in the human fibroblast system have been assembled to advance a working model for the mechanism by which LDL receptors cluster in coated pits^{1,31}. As described above, the existence of the internalisation mutation implies that the LDL receptor has two active sites—a binding site that must be on the external surface and an internalisation site that is postulated to be on the cytoplasmic surface. By analogy with other transmembrane proteins, the LDL receptor is likely to be synthesised on membrane-bound ribosomes and glycosylated in the Golgi apparatus (Fig. 5). It is then inserted into the plasma membrane at random sites. The receptor migrates laterally in the plane of the membrane until it reaches a coated pit. Interaction of the internalisation site with clathrin (or a clathrin-associated protein) fixes the receptor in the coated pit so that it is carried into the cell when the coated pit invaginates to form a coated vesicle.

Whether receptors reach the coated pits by diffusion in the plane of the membrane or whether they are actively propelled there is not known. Bretscher has suggested that the phospholipids of cell membranes continually flow towards coated pits and coated vesicles⁴⁴. This flow is envisioned to result from the insertion of lipids into the membrane at remote sites with resorption in coated pits and vesicles⁴⁴. If such a flow does exist, it could provide the force that carries selected receptor proteins to coated pits.

LDL receptors that enter cells in coated vesicles are not destroyed when the vesicles fuse with lysosomes. Rather, kinetic experiments suggest that the receptors return to the surface where they again cluster in coated pits; that is, they are recycled^{10,31}. This recycling phenomenon accounts for the fact that fibroblasts ingest saturating levels of LDL at a uniform rate for more than 6 hours even when synthesis of new receptors is blocked by cycloheximide. If recycling did not occur, all of the receptors would be consumed within the first 10 minutes after exposure to LDL.

Morphological and kinetic evidence indicates that insertion of receptors into the membrane, clustering in coated pits, internalisation, and recycling occurs continuously whether or not LDL is present^{2,19,31}. In each cycle the LDL receptor spends about 9 min on the cell surface. Assuming that LDL can bind to

the receptor at any time the receptor is on the surface, studies with LDL-ferritin indicate that in the steady state about one-third of receptors are diffusely distributed and two-thirds are clustered in coated pits^{2,19}. Thus, in each cycle the receptor spends about 3 min in a diffuse distribution on the surface and about 6 min in coated pits. The length of time between internalisation of the receptor and its reappearance on the surface is not known, but it must be shorter than the time the receptor spends on the surface because no appreciable pool of intracellular receptors is unmasked when binding assays are performed on broken cells⁴⁵. That membrane recycling can be extremely rapid has been shown by studies of the frog neuromuscular junction where the pre-synaptic coated vesicles recycle within seconds³⁷.

Other explanations for the morphological and kinetic data in fibroblasts are possible. For example, rather than being recycled it is conceivable that the LDL receptor never actually leaves the surface. That is, as the coated pit pinches off, the LDL is transferred from the receptor to some other carrier with the receptor escaping inclusion in the coated vesicle and thus remaining on the cell surface. While such a mechanism would be consistent with all current data, there is no direct evidence to support it.

The model for insertion of the LDL receptor into coated pits by lateral movement within the plane of the plasma membrane resembles an earlier model advanced for the assembly and secretion of lipid-envelope viruses in animal cells (reviewed in ref. 46). When the vesicular stomatitis virus replicates in cultured animal cells, it produces a messenger RNA for a transmembrane glycoprotein, the G protein, that is synthesised on host membrane-bound ribosomes and inserted at random

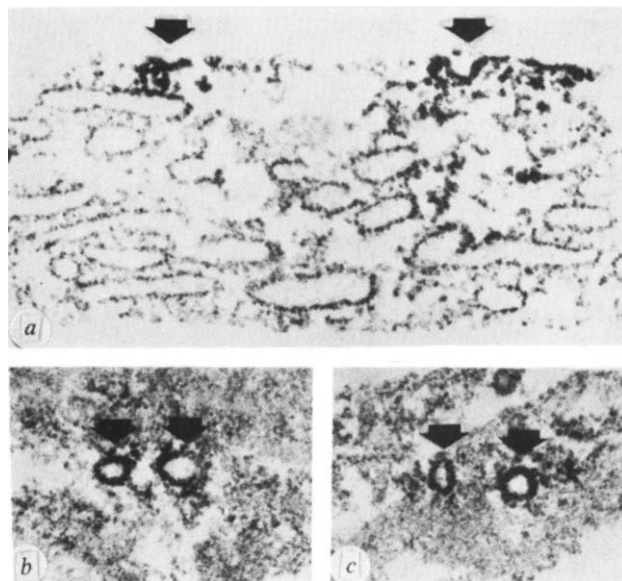


Fig. 3 Electron micrographs showing the localisation of clathrin in human fibroblasts as determined with the indirect immunoperoxidase technique. Monolayers of fibroblasts were fixed with 3% formaldehyde. The membrane was then made permeable with 0.05% Triton X-100, and the cells were incubated first with rabbit anti-coat protein γ -globulin (directed primarily against clathrin) and then with goat anti-rabbit IgG that was coupled to horseradish peroxidase. The cells were then stained for peroxidase as previously described²⁸. *a*, Peroxidase-positive indented regions of cell surface membrane (arrows). These regions have the typical shape of coated pits. The normal trilaminar appearance of the membrane has been destroyed by the Triton X-100 preparation procedure. *b*, and *c*, show peroxidase-positive endocytic vesicles (arrows) that have the morphology of coated vesicles. All micrographs are of unstained material. Magnifications: *a*, $\times 21,500$; *b*, $\times 20,000$; *c*, $\times 21,500$.

sites into the plasma membrane. The G proteins then gather together to form a patch of plasma membrane that ultimately becomes the envelope of the virus. This clustering of G proteins is mediated by their association with a cytoplasmic protein, the M protein, that is also coded by the viral genome but is synthesised on free ribosomes. The patch of membrane containing G and M proteins serves as the site of attachment of the viral nucleocapsid during viral budding. In the two models, the G protein is analogous to the LDL receptor, the M protein is analogous to clathrin, and the developing patch of viral envelope membrane is analogous to the coated pit. If the two working models prove to be correct, they would suggest a general principle: membrane structures such as viral patches and coated pits are assembled within the plane of the membrane by the interaction of transmembrane proteins with cytoplasmic organiser proteins.

Variations on a common theme

Table 1 lists the protein ligands for which receptor-mediated endocytosis has now been demonstrated. In each of these systems, uptake of the protein follows rapidly upon binding. In each case the endocytosis is carried out by the same receptor that is believed to mediate the physiologic action of the protein. For six of the systems, including the receptors for LDL^{2,19,20,27}, yolk proteins^{21,32}, transferrin²², epidermal growth factor²³, α -2-macroglobulin²⁴, and maternal immunoglobulins (IgG)^{21,47-49}, internalisation has been shown by electron microscopy to occur in coated pits and coated vesicles. In addition, evidence from the light microscope suggests that insulin enters mouse 3T3 cells through this route⁵⁰. In the other systems listed in Table 1, internalisation via coat pits and coated vesicles has not yet been visually demonstrated. However, in the case of transcobalamin II^{14,51}, chorionic gonadotropin¹⁵, and asialoglycoproteins^{16,17}, uptake rapidly follows binding, indicating a close coupling between the two events and in turn suggesting that coated pits may be involved.

In those systems for which coated pits have been documented, the mechanism for receptor clustering into the pits may vary. As described above, LDL receptors seem to be incorporated into coated pits by virtue of their internalisation sites in a process that is independent of LDL binding. One electron microscopic study of epidermal growth factor binding to human fibroblasts suggests that a significant proportion of these receptors behave similarly to those for LDL; even when epidermal growth factor was added in conditions in which lateral diffusion was inhibited by chilling the cells to 4 °C, 34% of the factor bound within coated pits, presumably to receptors that were already located there²³. On the other hand, light microscopic studies with fluorescent-labelled epidermal growth factor in mouse 3T3 cells suggest that most of the receptors are distributed diffusely when binding is conducted at 4 °C⁵⁰. Receptor clustering in coated pits was observed only after the cells were warmed to 37 °C in the presence of epidermal growth factor. Similar results were reported for fluorescent-labelled α -2-macroglobulin and insulin in the same cell type⁵⁰. The simplest, but not necessarily the correct, explanation of this discrepancy is that some receptors may reach coated pits as a result of a receptor-mediated mechanism, while others may require a ligand-mediated event.

In three of the systems in Table 1—LDL^{10,52}, transcobalamin II^{14,51}, and asialoglycoproteins^{16,53}—new receptors are efficiently regenerated, probably by recycling of internalised receptors, after the ligand is carried into the cell. As a result, cells internalise these ligands for hours at a steady rate. On the other hand, when epidermal growth factor is incubated with fibroblasts, the initial wave of receptor-mediated endocytosis leads to a rapid 80% depletion in the number of surface receptors^{13,53,55}. This reduction has been attributed to a degradation of the internalised epidermal growth factor–receptor complex, which is not accompanied by a reappearance of new receptors. In the continued presence of epidermal growth factor, cells establish a new steady state in which they bind,

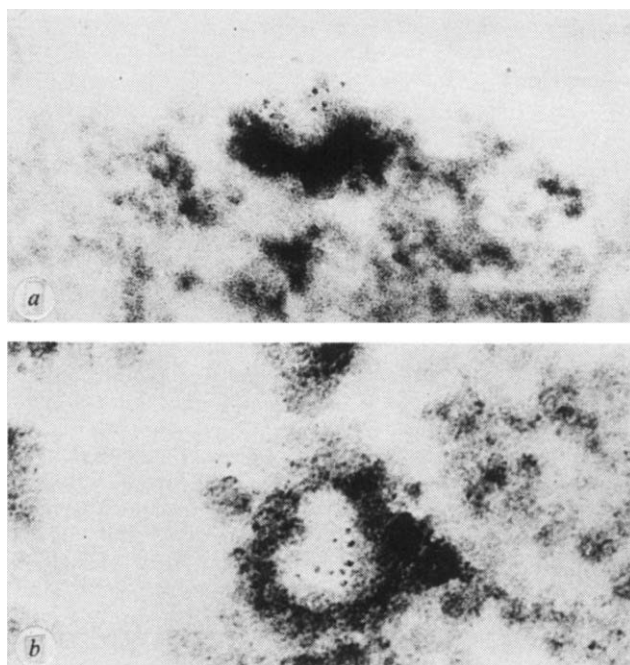


Fig. 4 Electron micrographs showing the binding of LDL-ferritin to clathrin-containing regions of cell membrane. Monolayers of human fibroblasts were chilled to 4 °C and incubated with LDL-ferritin at a concentration corresponding to 22 $\mu\text{g ml}^{-1}$ of LDL-protein for 30 min. The cells were then warmed to 37 °C for 8 min, washed at 4 °C to remove excess LDL-ferritin, fixed with 3% formaldehyde, and made permeable with 0.05% Triton X-100. The cells were then processed for indirect immunoperoxidase localisation of anti-clathrin binding sites as described in the legend to Fig. 3. *a*, Ferritin cores associated with an indented, peroxidase-positive segment of membrane that has the typical morphology of a coated pit, indicating that LDL-ferritin and the antibody directed against clathrin bind to the same region of surface membrane. *b*, Ferritin cores contained within endocytic vesicles that are rimmed by peroxidase reaction product that delineates the clathrin coat. All micrographs are of unstained sections. Magnification, $\times 100,000$.

internalise, and degrade the hormone continuously at about 20% of the initial rate. Whether receptor recycling occurs during this steady state is not known.

Whereas the coated pit-coated vesicle seems to be a common portal of entry for receptor-bound proteins, the ultimate destinations of these proteins vary. In seven of the systems in Table 1, the internalised protein (radiolabelled with ¹²⁵I in tyrosine residues) is rapidly degraded^{6,12-18,25,56-59}. In these systems, no significant amounts of radiolabelled products other than ¹²⁵I-monoiodotyrosine are formed by the cells. This suggests the concerted action of multiple proteases and thus implicates lysosomes^{56,57}. Moreover, in each of these systems, degradation of internalised protein is blocked by the lysosomal enzyme inhibitor chloroquine^{6,13-15,17,25,57}. For LDL^{2,52} and epidermal growth factor²³, lysosomal delivery has been demonstrated directly by electron microscopy.

Some of the proteins that enter cells through receptor-mediated endocytosis are not degraded but instead are directed to specific subcellular organelles. For example, yolk proteins accumulate in yolk granules apparently without degradation^{21,32}. Nerve growth factor, which enters the cell at the tip of the axon, migrates in vesicles to the cell body where it apparently accumulates in an intact form⁶². Although most of the receptor-bound insulin that enters cells is degraded in lysosomes^{12,25}, some fraction of receptor-bound hormone may be delivered to the Golgi apparatus⁶¹ and to nuclei⁶⁴. Lysosomal enzymes that are secreted by cells re-enter the cells through

receptor-mediated endocytosis and accumulate within lysosomes and Golgi-associated structures where they remain active for many days⁶⁵⁻⁶⁷. Finally, maternal immunoglobulins (IgG), which enter the fetal yolk sac or the neonatal intestinal epithelial cell at the apical surface, are transported intact in coated vesicles to the basal surface where the IgG molecules are discharged into the fetal or neonatal circulation^{21,47-49}.

An important question posed by the above findings relates to the mechanism by which endocytic vesicles and their contents are directed to specific delivery sites within the cells. Do proteins with different destinations enter cells in the same vesicles? If so, how are these proteins sorted and delivered to their separate destinations?

It should be pointed out that receptor-mediated endocytosis is not the only way in which proteins can enter animal cells. At least two other mechanisms exist. First, extracellular proteins are trapped nonselectively in fluid droplets that are internalised by cells through the invagination of noncoated regions of membrane⁷. To date, no receptor-bound protein has been demonstrated to enter cells through such noncoated vesicles. Second, some bacterial and plant toxins and certain viruses appear able to enter cells by direct penetration through the plasma membrane. Direct penetration of physiological molecules, such as protein hormones, has not yet been demonstrated.

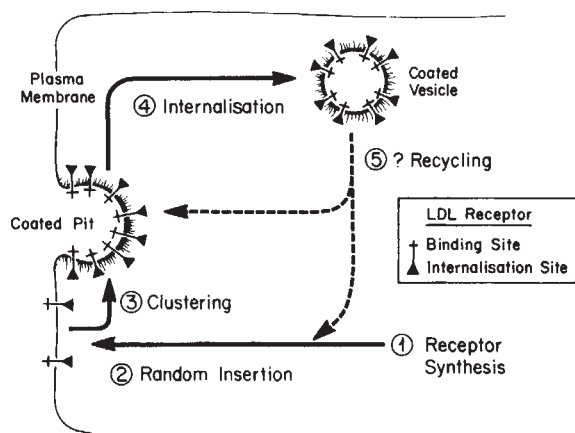


Fig. 5 Working model for the mechanism by which LDL receptors cluster in coated pits on the plasma membrane of human fibroblasts. The postulated steps are as follows: (1) synthesis of LDL receptors on polyribosomes; (2) insertion of LDL receptors at random sites along noncoated segments of plasma membrane, (3) clustering of LDL receptors in clathrin-containing coated pits, (4) internalisation of LDL receptors as coated pits invaginate to form coated endocytic vesicles, and (5) recycling of internalised LDL receptors back to the plasma membrane. It seems likely that the clathrin is also recycled and reutilised. Reprinted from ref. 31 with permission.

Why do cells need receptor-mediated endocytosis?

The studies cited in this review demonstrate that coated pits and coated vesicles function as transport organelles that carry selected receptor-bound proteins into cells in a tightly controlled fashion. It is easy to understand the need for such a system when cells are dealing with transport proteins such as LDL, yolk proteins, transferrin, and transcobalamin II. The reason for such a sophisticated mechanism of uptake for protein hormones is not yet clear. At the very least, the receptor-mediated uptake and degradation systems serve to degrade the protein hormones so that they, and in some cases their receptors, can act only once. But is this the only reason for receptor-mediated endocytosis of protein hormones? Does the endocytic uptake mediate any of the actions of the hormones? To date, no regulatory action of a

protein hormone (with the possible exception of nerve growth factor) has been shown to require endocytosis of the protein. Moreover, some events, such as insulin-mediated stimulation of glucose transport, occur too rapidly to be accounted for by endocytosis.

To date, only a few studies have addressed the question of whether the long-term biological effects of protein hormones require endocytic uptake. More systems are needed in which the entire sequence of hormone binding and hormone action can be studied by combined biochemical and morphological techniques in isolated cells. Such studies would be facilitated by the availability of cells bearing naturally-occurring or experimentally-induced mutations in the sequence between hormone binding and hormone action. With the process of receptor-mediated endocytosis now in focus and with the powerful tools of cell biology and somatic cell genetics at hand, it seems likely that such systems will be developed and that answers to the questions posed in this review will be forthcoming in the near future.

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articles

Non-exponential decay in dielectrics and dynamics of correlated systems

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A new model for the relaxation of a potential perturbation in dielectric materials is developed based on the correlated properties of a two-level system containing two types of decay mechanism. The time and frequency behaviour of the general relaxation process is shown to be in accord with recent experimental analyses. The technique developed to analyse the model is of general applicability in the solid, and liquid, state.

It has been shown¹ previously that for materials exhibiting a dielectric loss peak the magnitude of the imaginary part of the susceptibility, as a function of frequency, obeys the empirical relationship

$$\chi''(\omega) = \frac{\omega^m}{(\omega_p^{2s} + \omega^{2s})^{(1-n+m)/2s}} \quad (1)$$

where $0 \leq m, n$ and $s \leq 1$. In the post-peak region, $\omega > \omega_p$, this relationship can be written in the form $\chi(\omega) \propto (i\omega)^{-(1-n)}$ and Jonscher²⁻⁵ has suggested that the behaviour can best be understood as

$$\chi''(\omega)/\chi'(\omega) = \cot(n\pi/2) = \text{constant} \quad (2)$$

which states that the ratio of the energy lost to the energy stored is a constant. In the pre-peak region the imaginary part of the susceptibility exhibits a power law dependence, ω^m , and equation (2) is not obeyed. The empirical equation (1) describes a complete decay characteristic for the materials exhibiting loss peaks. It can be Kramers-Kronig transformed to give $\chi'(\omega)$, and agreement with experimental measurements has been observed. We use here the presence of a loss peak to define our term dipolar dielectric, and we shall only consider this class of materials.

The universal equation (2) requires the susceptibility to follow a power law in time (t^{-n}) in the equivalent, short time, region. It has recently been postulated by Ngai *et al.*⁶ that the origin of this form of decay lies in an infrared divergence mechanism and that

the basic requirements for the application of this mechanism in a wide range of systems have been established computationally.

Starting from the concept of an assembly in which the local units possess two equilibrium positions^{7,8} (a two-level system) we will show that the consequence of interaction between the local units leads to a complete description of the susceptibility of dipolar dielectrics. The post-peak behaviour arises naturally from this description. The method used is powerful and yields detailed and quantitative information on the microscopic structure and its dynamics. As the final expressions describe the macroscopic behaviour they can be conceived in a variety of ways and reveal the flexibility of the approach presented here. The results of this analysis have a wide range of applicability outside purely dielectric behaviour and similar approaches should be generally applicable.

The t^{-n} behaviour

Before describing dielectric relaxation in terms of the cooperative model we must describe briefly the origins and requirements of the t^{-n} behaviour, which is a special case of the time development of transients⁹. Consider a system divided into two interacting sub-systems. The first of these responds rapidly to a stimulus generating a change in the interaction which, in turn, causes a much slower response of the second sub-system. The state of the total system then corresponds to the excited first system together with the unresponded second system, and can be considered as a transient or metastable state which slowly decays as the second system responds. In this way a Franck-Condon progression is developed in molecular spectra¹⁰⁻¹², the fast system being a high frequency transition which is coupled to the slow response of a discrete spectrum of low frequency oscillations.

The special requirements for a t^{-n} behaviour are¹³: (1) a continuous spectrum of slow responders extending to zero frequency; (2) a constant density of transition states, per unit energy, for the slow system, in the same energy range; (3) an effective population distribution in the slow states such that they can be regarded as either fully occupied or unoccupied only.

The time behaviour of the state vector of the transient can be obtained from the total hamiltonian H as

$$\exp(-iHt) = \langle t|0 \rangle \quad (3)$$

which in second order perturbation theory^{10,14}, in energy/frequency normalised units, becomes

$$\exp(-iEt) \cdot \exp\{-F(t)\} \quad (4)$$

with E the unspecified excitation energy of the transient, and

$$F(t) = iN\zeta t - \int_0^t \frac{|VN|^2}{u} \{1 - \exp(-iut)\} du \quad (5)$$

N is the total number of slow responders and ζ is their maximum excitation energy, the constant density of states is thus N/ζ . V is the interaction change brought about by the fast excitation and u the energy difference for nett excitations within the slow response system. Equation (5) is obtained by allowing V to cause excitations and de-excitations in the slow system, the double sum over initial and final states being replaced by the already completed sum over initial states, for a given energy difference u , and an integration over u . This is shown in Fig. 1.

The average energy change per fast excitation will be distributed over the N slow responders and is NV . As ζ is the maximum excitation energy, $|NV|^2/(\zeta)^2$ is less than unity and is set equal to n . Integrating equation (5)^{13,15,16} gives

$$F(t) = iN\zeta t - n\{\gamma + \ln(i\zeta t) + E_1(i\zeta t)\} \quad (6)$$

where γ is Euler's constant and $E_1(iz)$ an exponential integral¹⁷. For short times, such that $i\zeta t < 1$

$$F(t) \rightarrow 0 \quad (7)$$

and the transient oscillates with its excitation frequency corresponding to the energy E . At long times ($i\zeta t > 1$)

$$F(t) = \exp\{-i(E - n\zeta)t\} \cdot \exp(-n\gamma) \cdot \exp(i\pi/2) \cdot (i\zeta t)^{-n} \quad (8)$$

where the excitation energy of the transient has been reduced by $n\zeta$, and the transient decays as t^{-n} .

In applying this result to our problem the fast excitations and the slow responders are assumed to lie in the same continuum of states, the slow responders merely being slower than a particular fast transient excitation within the same total system. The system returns to its unexcited state by means of excitations of the slow responders, which decay monotonically. For this reason E is taken equal to $n\zeta$, and it has already been shown that n lies between zero and unity corresponding to no excitation or total excitation of the system respectively. Note that any observable property of the transient follows a behaviour governed by the real part of equation (4), and thus oscillates with diminishing frequency while decaying as t^{-n} .

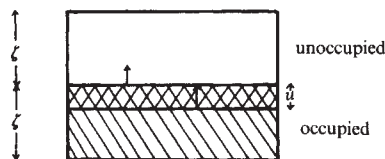


Fig. 1 The two-level system: the range of initial states available for excitation with energy u is shown by cross hatching. The equivalent number of initial states is (Nu/ζ) where N/ζ is the constant energy density.

Interacting two-level systems

A local two-level system can be considered as a group of atoms, ions or molecules possessing two equilibrium configurations separated by a small energy difference and between which local tunnelling is allowed^{7,8}. Such a system can be described by a spin

formalism in which the energy tensor is

$$\begin{pmatrix} B_e & U_e \\ U_e & -B_e \end{pmatrix} \quad (9)$$

where B_e is half the energy difference between local potential minima and U_e is the off-diagonal tunnelling (transition) element. Such systems can couple to elastic deformations and have been used to describe a range of glass properties¹⁸. When the local system possesses a dipole moment the coupling to an electric field takes the form

$$\begin{pmatrix} d & \mu \\ \mu & -d \end{pmatrix} F \quad (10)$$

with the off-diagonal elements μ allowing a resonance absorption, the diagonal elements a relaxation spectra. A spin-spin interaction arises from the coupling of pairs of local spin systems through the off-diagonal elements of the tensor in equation (9). These interact with phonons and give the usual dipolar spin-spin interaction through a virtual two-phonon exchange mechanism¹⁹. The spin-spin interaction can be regarded as a perturbation on an unperturbed local system with energy levels $\pm B_e$ together with a local transition interaction. It contains three types of term, each of which has a definite and unique influence on the overall system.

(1) The secular interaction: this is a dipole-dipole cooperative interaction which contributes to the energy of each unperturbed spin. It is the basis of the Ising hamiltonian and when calculated in the mean field approximation has a contribution to B of $T_c M$, where M is the mean value of the z component of the local dipole unit vector, in this case electric, and T_c is a characteristic parameter of the system. The system can no longer be regarded as a set of local systems because of the cooperative interaction. Its energy levels are macroscopic and the macroscopic thermal average, M_e , of M is

$$M_e = \tanh\left(\frac{B + kT_c M_e}{kT}\right) \quad (11)$$

which is a consequence of the condition that the dipoles can only be in one of two states. B is the average of B_e over all the system, and the dipole of the system in equilibrium is $NM_e d$. The value of M_e is defined by equation (11) and is determined from B , T_c and the temperature T . The energy B can be regarded as the splitting of a two-level system by a well-defined internal field. In amorphous glassy systems the local value of B_e is not constant but takes a continuous range of values with a constant number density at each value^{19,20}. It has been suggested⁶ that this applies to a wide variety of materials.

(2) The flip-flop interaction: this interaction allows a pair of dipoles to exchange spins synchronously and without altering M . Local spins move through the whole system by this mechanism. The time taken to complete a spin exchange is

$$t_e \approx \pi / V_{ff} \quad (12)$$

where V_{ff} is the flip-flop interaction energy. The time has been estimated as 10^{-8} – 10^{-10} s (ref. 19).

On a time scale greater than t_e the local spins will sample the whole of the local values of B_e . In general those local spins whose values of B_e differ by less than the maximum value of the interaction energy, $V_{ff \max}$, can be regarded as in resonance and hence involved in a true spin-spin exchange. Those spins with a greater difference than $V_{ff \max}$ interact in an off resonance manner and do not have a true spin-spin exchange.

In this way, on a time scale greater than t_e , the macroscopic value of B in equation (11) will alter without altering M_e . But the nature of equation (11) requires an alteration in B to generate a consequent change in M_e . Thus the flip-flop interaction has to be regarded as causing fluctuations in M_e about a well defined average.

(3) The raising and lowering interaction: this interaction couples M with raising and lowering operators. These operators allow excitation and de-excitation of the unperturbed local

system, and generate absorptions at multiples of the Larmor frequency in nuclear magnetic resonance spectra²¹. When paired together the value of M is unchanged and oscillations are generated at frequencies equal to the difference between excited and unexcited local systems²². Since the frequencies span the range from zero up to a particular maximum they can be regarded as a system of slow responders.

All spins will be connected by this interaction, hence a fluctuation such as a flip-flop interaction will create a transient which decays as t^{-m} as the frequencies less than $V_{ff\max}$, that is, 10^8 – 10^{10} Hz, respond. The magnitude of the parameter being given by

$$m = (V_{ff\text{ average}}/V_{ff\max})^2 \quad (13)$$

The rate equation

The fundamental concepts required to establish the framework of our approach have already been established. The basic model is that a deviation from equilibrium in a macroscopic system resulting from the application or removal of an external field is restored to an equilibrium that is itself fluctuating on a long time scale. Because of the connection between M and B in equation (11) fluctuations in the ground state energy B must affect the rate of restoration of equilibrium. Calculations using this model are applicable to many fields but we shall consider here a dipolar dielectric.

The dipolar dielectric is represented by a double minimum in the total (macroscopic) free energy as indicated in Fig. 2. Each minimum refers to a set of configurations of local dipoles with their orientations effectively in one of two alternative directions. Each configuration is thus specified by a set of local dipole orientations, simultaneously fully occupied, which have an equal number of unoccupied levels belonging to a configuration with an opposing dipole orientation and separated from them by a large potential barrier. The two sets of configurations are therefore only accessible to each other either by thermally activated or tunnelling processes. Thermal equilibrium is established between groups of macroscopic configurations rather than independent local orientations.

After the removal of an externally applied perturbing influence there are two competing relaxation processes as well as the fluctuations to consider. These are thermal activation and local tunnelling, (Fig. 2c and b).

The rate equation for the thermal process can be written as²³

$$\frac{dM}{dt} = -\nu_E \cosh\left(\frac{B + kT_c M}{kT}\right) \left\{ M - \tanh\left(\frac{B + kT_c M}{kT}\right) \right\} \quad (14)$$

where ν_E has an activated form with a pre-exponential frequency of the order of 10^{13} Hz, that is

$$\nu_E = \nu_0 \exp(-\Delta/kT) \quad (15)$$

Writing

$$M = M' + \tanh\left(\frac{B + kT_c M_e}{kT}\right) = M' + M_e \quad (16)$$

a linear form of equation (14), exact in M_e , can be found,

$$\begin{aligned} \frac{dM'}{dt} &= -\nu_E \cosh\left(\frac{B + kT_c M_e}{kT}\right) [M' \{1 - (1 - M_e^2) T_c/T\}] \\ &= -\omega_p M' \end{aligned} \quad (17)$$

where M' is the deviation from equilibrium of M .

As ω_p involves M_e it can be considered as an expectation value of an operator in the fluctuating system. Thus the rate itself fluctuates and must be averaged over these fluctuations in ω_p . To carry out this averaging, the form of the equilibrium fluctuations must be determined.

Resonance flip-flops exchange dipoles between the two minima, in each of which a new configuration is generated. The initial equilibrium value of B of the macroscopic state is not appropriate to the new configurations and the state evolves in

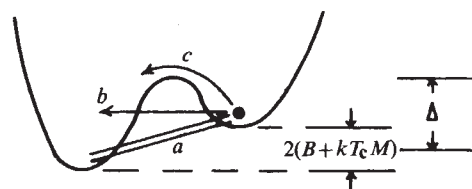


Fig. 2 A double minima potential. *a*, A tunnelling process of synchronous excitation and de-excitation. *b*, A cooperative tunnelling relaxation process. *c*, A thermally activated relaxation process. Δ , the average energy of the maximum above the two minima.

time to accommodate the change. The state of the configuration in each minimum immediately following a flip-flop will effectively be that due to the excitation of a dipole from a fully occupied to a non-occupied level. The population criterion (3) for the slow responders is completely fulfilled and the time evolution of each configuration, and hence the system, follows a time power law, the exponent of which has been defined as m .

During the transient decay the requirement of a well defined average necessitates a balance which arises from the multiply-connected pairs between the two configurations of the transient, generating a new equilibrium state of the system that satisfies equation (11).

The fluctuations must therefore follow a time development of the form

$$(t - t_1)^m \cdot t_1^{-m} \quad (18)$$

as the balancing is delayed in time by t_1 to allow for the initial decay. Equation (18) represents fluctuations about a well defined average expectation value because of the constant time average

$$t^{-1} \int_0^t (t - t_1)^m \cdot t_1^{-m} dt_1 = \Gamma(1 + m) \cdot \Gamma(1 - m) \quad (19)$$

To allow for these fluctuations a time average has to be taken in the rate equation, equation (17),

$$\left\langle \frac{dM'}{dt} \right\rangle \propto -\omega_p(t)^{-1} \int_0^t (t - t_1)^m t_1^{-m} M'(t - t_1) dt_1 \quad (20)$$

The evolution of the initial state, t_1^{-m} , competes with the thermal decay process, but the balancing $\{(t - t_1)^m\}$ initiated by the decay at t_1 generates a new macroscopic state satisfying the initial conditions and thus initiates a new contribution to the relaxation, giving the composite relaxation rate at time t shown in equation (20). The value of $M'(t - t_1)$ is evaluated from

$$\frac{d\{M'(t_s - t_1)\}}{d(t_s - t_1)} = -\omega_p(t_s - t_1)^m t_1^{-m} M'(t_s - t_1) \quad (21)$$

giving

$$\ln[M'(t_s)/M'_{(0)}] = -\omega_p \int_0^{t_s} (t_s - t_1)^m t_1^{-m} d(t_s - t_1) \quad (22)$$

and hence, using equation (20), and normalising to unity,

$$M'(t - t_1) = M'_{(0)} \exp\{-\omega_p(t - t_1)\} \quad (23)$$

The variable in equation (21) is the time span, $(t_s - t_1)$, during which the relaxation proceeds uninterrupted as opposed to the actual time, t , at which measurements are made.

The initial deviation from equilibrium, $M'_{(0)}$, arises from the removal of a perturbation which alters B , for example an external electric field which makes a contribution Fd to B where d is the local z component of the dipole moment, and is given by

$$M'_{(0)} = \tanh\left(\frac{B + Fd + kT_c M'_{(0)}}{kT}\right) - M_e \quad (24)$$

That is, F rotates the macroscopic dipole towards or away from the fixed internal field direction.

The rate constant ω_p is the maximum probability that a thermally activated process leads to a transition of a dipole between alternate minima. The activated factor is the probability for a thermal process of sufficient energy to surmount the barrier, and the pre-exponential factor is the quantum mechanical transition rate at the barrier peak.

A second independent relaxation process, that of local tunnelling, may also occur. Each tunnelling event takes place in a time of approximately ν_0^{-1} and flips a dipole from one minimum to the other. A transient configuration is thus created in each well in a manner similar to that generated by flip-flops, and which decays by a time power law with a different exponent,

$$\cos(n\pi/2) \cdot (t\zeta)^{-n} \quad (25)$$

the range of frequencies, ζ , in the slow system being from zero up to ν_0 , or the maximum value in the system, whichever is lower. This behaviour describes the tunnelling relaxation of a deviation in M , and in this case there is no balancing time behaviour. The magnitude of n is proportional to the amount of configuration change introduced by exciting a single dipole, as a fraction of the maximum possible change, and is therefore the degree of cooperation of dipole tunnelling.

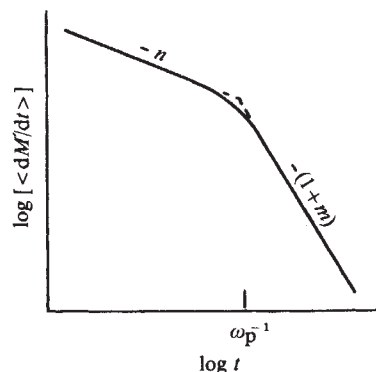


Fig. 3 A log-log plot of the decay current as a function of time. The power law limiting behaviour regions can be clearly seen.

This cooperative tunnelling relaxation process competes independently with the thermally activated relaxation, equation (23), and takes place on the same time scale. Therefore, at a time $t-t_1$ after the relaxation has been initiated at t_1 , M' has relaxed to

$$M'(t-t_1) = M'_{(0)} \cos(n\pi/2) \cdot [\zeta(t-t_1)]^{-n} \exp\{-\omega_p(t-t_1)\} \quad (26)$$

The value of the relaxation current observed at time t is that given in equation (20) with $M'(t-t_1)$ given in equation (26) and is

$$\begin{aligned} \left\langle \frac{dM'}{dt} \right\rangle &= - \frac{\omega_p \cos\left(\frac{n\pi}{2}\right) \zeta^{-n} \int_0^t (t-t_1)^m t_1^{-m} M'_{(0)} (t-t_1)^{-n} e^{-\omega_p(t-t_1)} dt_1}{\int_0^t (t-t_1)^m t_1^{-m} dt_1} \\ &\propto -\omega_p \cos\left(\frac{n\pi}{2}\right) \zeta^{-n} M'_{(0)} e^{-\omega_p t} \cdot t^{-n} {}_1F_1(1-m; 2-n; \omega_p t) \end{aligned} \quad (27)$$

where ${}_1F_1(;;)$ is the confluent hypergeometric function²⁴. Equation (27) describes a complex situation. An initial deviation decays through two independent processes. One is an internal readjustment, and is described by the t^{-n} behaviour. The other is a thermal decay process in which the rate constant fluctuates about an average value. These fluctuations are the result of a

second microscopic interaction. The composite rate observed at time t , therefore, has to be averaged over the fluctuations. A linear Ising model calculation of configuration correlation functions²⁵ has revealed some of the features of the above expression.

Frequency-dependent susceptibility

To obtain the linear frequency-dependent susceptibility a form of $\langle dM'/dt \rangle$, equation (26), requires to be determined in which the initial deviation from equilibrium is linear in field strength. Expanding equation (24) gives

$$M'_{(0)} = \frac{Fd}{kT} (1-M_e^2) \{1 - (1-M_e^2)T_c/T\}^{-1} \quad (28)$$

The frequency dependence of the susceptibility is given by a standard transformation of equation (26). The time development of equation (26) is shown in Fig. 3. At short times the confluent hypergeometric function has a limiting value of unity, as has the exponential term, and hence

$$\left\langle \frac{dM'}{dt} \right\rangle = J \propto (\omega_p t)^{-n} \quad (29)$$

where J is the decay current. At infinite time the asymptotic form of the hypergeometric term is proportional to $\exp(+\omega_p t) \cdot (\omega_p t)^{n-m-1}$ and the equivalent current is given by

$$(\omega_p t)^{-(1+m)} \quad (30)$$

In the limited region around $t \approx \omega_p^{-1}$ the decay is dominated by the exponential term. The Laplace transform of equation (26) is²⁶, in normalised form,

$$\chi(\omega) \propto \frac{\omega_p^{1-n}}{(\omega_p + i\omega)^{1-n}} \cdot {}_2F_1\left(1-n, 1-m; 2-n; \frac{\omega_p}{(\omega_p + i\omega)}\right) \quad (31)$$

in which ${}_2F_1(;;)$ is the gaussian hypergeometric function²⁶. Equation (31) has the simple asymptotic behaviour that at frequencies greater than ω_p both the real and imaginary parts of the complex susceptibility are proportional to $\omega^{-(1-n)}$, in agreement with equations (1) and (2). At frequencies less than ω_p the imaginary part of the susceptibility is proportional to ω^m and the real part is given by $\chi'_{(\omega=0)} - A\chi''_{(\omega)}$ where A is a constant, in agreement with equation (1). The curvature parameter s of equation (1) is found to be a single-valued function of m and n .

The particular value of m equal to unity is of interest as it can arise either when there is perfect correlation in the flip-flop processes, or when measurements are made at sufficiently high frequencies that the slow responders cannot give rise to the fluctuation process. Both forms have been observed experimentally¹.

Discussion

The basic requirements for the present model is the presence of a set of two-level systems in which the frequency difference ranges from zero upwards, and in which the number density is effectively constant. The existence of these states has been demonstrated experimentally in glasses¹⁹ and theoretically established in other materials⁶; from this the high frequency behaviour follows. It should be pointed out that most dielectric susceptibility measurements are made close to a phase transition^{27,28} where a cooperative system of structural changes with at least two local potentials must exist. The local two-level system considered here can be regarded as describing the structural changes involved in a phase transition. The processes described here are the microscopic reality behind Jonscher's 'screened hopping model'²⁹. The exponents n and m have been determined as follows; m is the degree of structural adjustment required for the average flip-flop process. It is therefore a correlation of these processes in the ground state. n is the degree

of structural adjustment required for the average spin flip (spin raised or lowered). It is thus a correlation factor for the dynamic restorative tunnelling events.

It is expected that mechanical strain, nuclear magnetic resonance T_1 measurements³⁰, magnetic susceptibility and the molecular dynamics of plastic crystals will show similar behaviour²⁸. In particular ultrasonic absorption measurements in which the pseudo-spins respond to variations in B produced by acoustic vibrations show a similar response³¹.

Finally we point out that a non-exponential decay behaviour is a theoretical necessity in all systems which do not have an infinite range of energy in their decomposed state³², for example which may recombine. The required behaviour has to be faster

than exponential at short times and slower than exponential at long times. The transitional behaviour between these regions is close to exponential in form, as it is here. Usually the available experimental time region is such that only minor deviations from an exponential behaviour can be observed. Because dielectric susceptibility is observable over wide ranges of frequency and amplitude, and is amenable to temperature scaling, it provides a unique opportunity to study the details of the non-exponential decay and the microscopic mechanisms involved.

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Thermal aspects of komatiite generation and greenstone belt models

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Thermal modelling suggests that the problems posed by the high liquid temperatures (~1,650 °C) of peridotitic lavas in Archaean greenstone belts, and the implied high degree of mantle melting (~70%), are significantly reduced by considering uprise of a more refractory mantle diapir having an inherent density contrast with the surrounding mantle, and in a tectonic environment analogous to a marginal basin.

LAVAS of komatiitic composition^{1,2}, representing very high magnesian liquids with up to 33% MgO, are a common and distinctive magma type in Archaean greenstone belts, although comparable high magnesian liquids become increasingly rare in younger post-Archaean volcanic provinces. They occur most frequently in the lower parts of greenstone volcano-sedimentary sequences, where there may be several cycles of ultramafic-mafic lavas. Although crystal fractionation has been demonstrated in some ultramafic-mafic lavas³, the presence of spinifex quench textures⁴ indicates that in most cases the high-MgO character is primary and not due to olivine accumulation. Deriving such highly magnesian liquids by mantle fusion poses severe thermal problems.

Experimental studies⁵ have shown that the high liquidus temperatures of peridotitic komatiite (~1,650° at 1 atm) would require ~70% partial melting of mantle pyrolite. Similar melting experiments on mantle nodules⁶ have also demonstrated

that high degrees of melting would be required to produce peridotitic komatiite liquids. Modelling of the thermal evolution of ascending pyrolite diapirs^{7,8} indicates that attainment of this high degree of partial melting would necessitate initiation of mantle diapirs from depths in excess of 300 km. This somewhat extreme requirement has led to suggestions⁸ that the mantle source regions may have been enriched in radioactive heat-producing elements.

Recent detailed geochemical studies of Archaean greenstone volcanic sequences in Canada, Australia, Rhodesia, South Africa and Finland⁹⁻¹² have shown, however, that many peridotitic komatiite lavas not only have low trace element abundances, as may be expected with high degrees of mantle melting, but also have light rare-earth depleted rare-earth element patterns and low incompatible element abundances (Fig. 1). These features have been taken as indicating that such peridotitic komatiites are derived from a 'depleted' mantle source¹² similar to that for modern mid-ocean ridge basalts, and are most unlikely to have been enriched in U, Th, K and Rb. Other peridotitic komatiites may have essentially undepleted or even slightly enriched geochemical characteristics¹¹, but these are not a dominant group. The geochemical studies have also demonstrated that associated tholeiitic basalts in greenstone sequences have flat chondritic rare-earth patterns (Fig. 1), higher levels of incompatible elements and essentially 'undepleted' incompatible element ratios. They do not seem to be consanguineous with the depleted komatiitic lavas but have been derived from a different mantle source. Inhomogeneity in the Archaean mantle

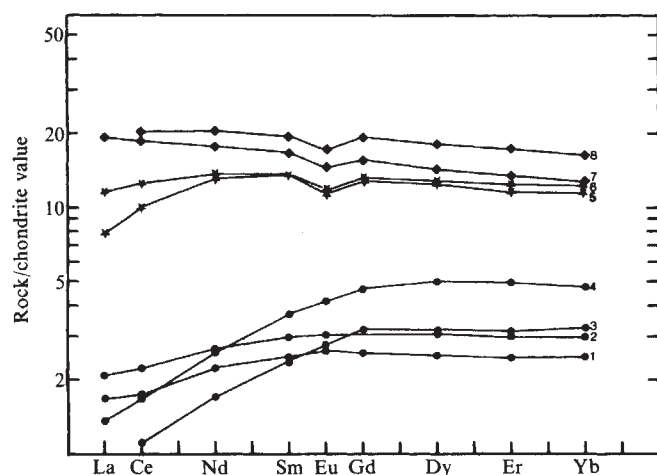


Fig. 1 Selected rare-earth patterns of depleted peridotitic komatiite (●); basaltic komatiite (★); and tholeiitic lavas (◆) from Australian, Rhodesian and North American greenstone belts. Data sources: Patterns 1, 2, 4, 5, 6, and 7 ref. 11; pattern 3, ref. 9; pattern 8, ref. 36. See also ref. 12.

is indicated^{11,12}. The thermal problem of accounting for the high degree of melting of the more depleted mantle source is, however, exacerbated.

Thermal aspects

Cawthorn's⁸ modelling of the thermal budget of ascending mantle diapirs was based on estimated thermal parameters for pyrolite (undepleted mantle). We have repeated these calculations using a depleted, more refractory peridotite (which has had a basalt fraction extracted), and the results are shown in Table 1. A depleted, more magnesian peridotite not only has a higher 1-atm solidus temperature, but also has higher values than pyrolite for the latent heat of fusion and for the heat capacity. Estimates of these quantities have been made, and although the absolute values for the chosen parameters are uncertain, they do illustrate the relative difference in melting behaviour of a pyrolite and a more refractory mantle diapir. As emphasised by Cawthorn⁸, the latent heat of fusion exerts a large buffering effect on the degree of mantle melting. In fact, on the basis of recent thermochemical data¹³, the values used by Cawthorn for the latent heat of fusion may be rather low, accentuating the thermal problem of deriving komatiitic liquids from pyrolite diapirs. Similarly the value assumed for the latent heat of fusion of depleted mantle may be an underestimate, but was chosen only to be of the correct magnitude for direct comparison with Cawthorn's modelling.

For both models the mantle source region is assumed to be at the same initial temperature. Note, however, that the more magnesian depleted peridotite would be of lower density and significantly more buoyant than the pyrolite¹⁴, and may even begin to rise at temperatures below that of surrounding and overlying undepleted mantle¹⁴. Because the depleted peridotite solidus is 200 °C higher than that of pyrolite, a depleted peridotite diapir must undergo more adiabatic uprise in the solid state before intersecting the solidus than would a pyrolite diapir derived from the same depth. This has been accommodated in the modelling. Nevertheless, Table 1 shows that a refractory mantle model allows a higher 1-atm liquid temperature with a smaller degree of partial melting than does the pyrolite model; moreover, the Mg contents of the erupted liquids (calculated on the basis of the method of Hanson and Langmuir¹⁵) are also higher.

The depleted peridotite model therefore makes it possible to obtain the required liquidus temperature (~1,650 °C) by initiation of diapirism from shallower depths and with a smaller degree of partial melting than allowed by the pyrolite model. At the same time the depleted peridotite model has a built-in buoyancy factor able to initiate diapirism. Whether these features can be reconciled with a viable tectonic model for greenstone belts remains to be determined.

Tectonic models

In seeking an acceptable tectonic model for greenstone belt formation it is necessary to account for the field, tectonic and chronological relationships of the various rock types, the geochemical characteristics of the lava sequences and associated plutons, and the thermal problems of komatiite generation. A wide variety of uniformitarian and non-uniformitarian models have been proposed^{1,16-22}, although few consider the thermal constraints on the generation of komatiites. On the basis of heat flow modelling, Bickle²³ has suggested that subduction did occur in the Archaean, with sub-lithospheric heat flow of two or three times the present values and involving rapid production of lithospheric plates of comparable thickness to those at present. If subduction did take place, then the depleted mantle cap to the subducting lithosphere is an obvious source of depleted refractory mantle with the potential for diapiric uprise. Indeed Oxburgh and Parmentier²⁴ have suggested this as a probable mechanism for back-arc spreading and the formation of marginal basins. Here we extend this concept to link with earlier proposed marginal basin models for greenstone belts²².

The essence of the Oxburgh and Parmentier^{14,24} model is that the depleted lithosphere with its basaltic capping sinks into the mantle until it heats up to or near the temperature of the surrounding mantle. At this point it segregates from the dense eclogite cap and rises diapirically because there is a significant

Table 1 Ascending peridotite diapirs: % partial melting and temperatures and MgO contents of liquids

Initial P, T and depths of diapir			Pyrolite model ⁸			Depleted peridotite model		
P (kbar)	Depth (km)	T (°C)	Final T (°C)	% Melt	% MgO*	Final T (°C)	% Melt	% MgO*
50	150	1,700	1,372	29	21	1,513	19	31
75	225	1,950	1,497	47	32	1,651	42	44
100	300	2,200	1,606	69	42	1,780	63	53

Parameters used in the modelling are:

Heat capacity of solid (cal g⁻¹ °C⁻¹)
Heat capacity of liquid (cal g⁻¹ °C⁻¹)
Latent heat of fusion (cal g⁻¹)

Pyrolite model ⁸	Depleted peridotite model
0.30	0.35
0.50	0.55
100	120

Both models assume an adiabatic cooling rate of 1 °C kbar⁻¹, with gravitational heat loss retained in the diapir of 0.4 cal g⁻¹ kbar⁻¹. In the pyrolite model⁸ the pyrolite solidus is taken to be at 1,200 °C at 1 atm, with dT/dP of 10 °C kbar⁻¹ with a nonlinear melting interval. The depleted peridotite model assumes a solidus at 1 atm, of 1,400 °C, again with dT/dP of 10 °C kbar⁻¹ but with a constant linear melting interval of 600 °C. Higher heat capacity and latent heat of fusion values based on mineral data in ref. 13.

* Modelling of MgO abundances in liquids similar to that of Hanson and Langmuir¹⁵, using 1-atm distribution coefficients. Note quoted MgO values are in cation mol %. Pyrolite⁷ has 48 cation mol % MgO; depleted peridotite assumed to have 53 cation mol % MgO (for reference, sample 49J from the Barberton belt^{1,5} has 32% MgO equal to 42 cation mol %).

density difference ($0.06\text{--}0.09\text{ g cm}^{-3}$) from the surrounding more Fe-rich undepleted mantle. The rising diapir will at some time intersect the refractory mantle solidus and thereafter undergo progressive fusion. The rising diapir may cause fusion of the normal mantle surrounding it (this is especially true where intrinsic buoyancy is a significant driving force in diapiric uprise²⁵), allowing potential mixing of mantle sources or magmas. Thus the geochemistry of the erupted magmas could conceivably vary from 'depleted' to 'enriched' and need not directly reflect the composition of the diapir itself. The first magmas erupted would be peridotitic komatiite, but perturbation of the mantle by the diapir and dissipation of its remaining thermal energy would cause extensive basaltic volcanism characteristic of many greenstone sequences. Because this type of diapirism is closely linked to subduction and hence to zones of crustal generation, the general association of rock types would be similar to that proposed for the *rocas verdes* marginal basin model of Tarney *et al.*²².

The present model, however, allows considerable improvements to be made to the *rocas verdes* model, particularly towards explaining the lack of rifting of the lithosphere (and consequent spreading), the occurrence of multiple greenstone belts, repetitions of the volcanic cycle and the lack of modern equivalents of komatiitic lavas.

An important feature of the model is that the driving force is the intrinsic density contrast rather than a thermally dependent density contrast. The rate of diapiric uprise depends on both the size of the diapir and the viscosity of the surrounding mantle. High heat flow in the early Archaean²³ would mean that the mantle had a low viscosity, thereby enabling smaller diapirs to rise, with perhaps more than one pulse, leading to repeated volcanic sequences. However, the viscosity of the mantle will increase with time as the geothermal gradient falls as a consequence of crustal growth and the transfer of radioactive elements U, Th, K and Rb into the crust. Conversely the upper mantle becomes progressively more depleted and refractory (as evidenced by Nd and Sr isotope data²⁶), lessening the density contrast with the subducting lithosphere. Thus only increasingly larger diapirs would be able to rise. The implication is that earlier greenstone belts might be smaller (for example, Barberton and Pilbara greenstones) with perhaps more repeated volcanic cycles, whereas later belts would be larger and more linear (for example, Superior Province, Yilgarn Block). Small diapirs might thin the crust and promote basin formation, but would not necessarily rupture the lithosphere. Eventually the density contrast would become too small and the viscosity too high to allow refractory mantle diapirs to migrate to higher levels. A corollary of this is that there may be no (or very rare) modern equivalents of komatiitic lavas. Alternatively, only very large diapirs would be able to rise; these, however, would rupture the lithosphere and generate seafloor spreading similar to that in modern back-arc marginal basins. It is interesting that high-magnesian (up to 18% MgO) lavas and dykes are known from several marginal basin ophiolite complexes, including the *rocas verdes* complex in southern Chile²⁷.

The existence of multiple greenstone belts in younger Archaean provinces (for example, Superior Province, Yilgarn Block) poses problems for the conventional marginal basin model, bearing in mind the time constraints from these areas. Recent isotopic studies²⁸ have indicated that most of the Superior Province, including the greenstone belts, formed within a restricted time span of 100–200 Myr. This implies extremely rapid crustal generation and penecontemporaneous formation of the greenstone belts. Similar evidence exists from the Yilgarn Block²⁹. One possibility is that the subduction zone migrates oceanwards as the newly generated crust accretes laterally and that a series of greenstone belts are formed in sequence, each generated by individual diapiric events. A modern parallel is the Mariana Arc system where a series of remnant arcs and intervening marginal basins have been formed since the mid-Tertiary as the active arc itself has moved eastwards³⁰.

However, it is also possible that there is a small but significant hiatus between the generation of crust and formation of greenstone belts, and that the latter were all formed simultaneously. Cox³¹, for instance, has used the Oxburgh–Parmentier model²⁴ to account for the extensive early Mesozoic Karroo volcanism in southern Africa. He suggests that continued subduction of Pacific lithosphere along the western margin of Gondwanaland during the late Palaeozoic led to a substantial build-up of low-density refractory mantle in the 'back-arc' region beneath the continent, and that it was the eventual diapiric rise of this material into the more fertile mantle above that provided the thermal energy for the extensive Karroo-related volcanism. For this to be a feasible mechanism requires a fairly shallow subduction zone in order to carry depleted lithosphere well beneath the continent. Shallow subduction beneath the Andes is associated with voluminous calc-alkaline volcanism and plutonism, while equivalent subduction beneath the active basaltic island

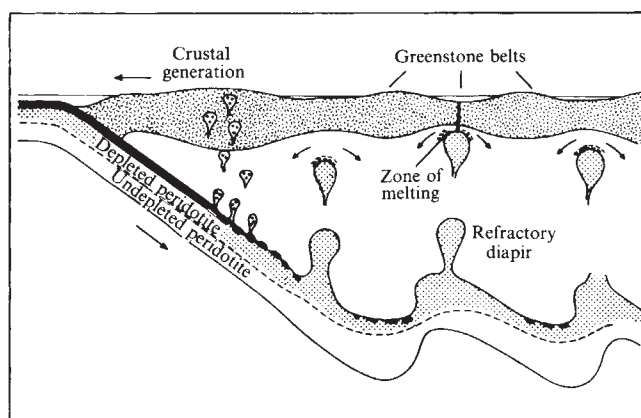


Fig. 2 Schematic representation of back-arc diapirism in a region of rapid lateral crustal growth. Possible model for multiple greenstone belts is shown.

arcs and marginal basins of the Western Pacific is located along steeply inclined Benioff zones. Molnar and Atwater³² have ascribed this difference to the age of the subducting lithosphere: older, cooler lithosphere in the Western Pacific sinking back steeply into the mantle while younger warmer lithosphere subsides more gently. The high heat flow in the Archaean coupled with rapid plate production and shorter convection cells would favour shallow subduction and, by analogy with the Andes, extensive crustal generation. The refractory subducted lithosphere could rise diapirically at several points under a continent to produce multiple (and ensialic) greenstone belts (Fig. 2). Provided they originated at depths in excess of 200 km, they could generate high-temperature lavas with the chemistry of peridotitic komatiites, as our calculations have shown.

Jordan³³ has argued, from seismic and heat-flow evidence, that the sub-continental lithosphere is composed of much more refractory mantle than the oceanic lithosphere, and that the thickness of this subcontinental 'tectosphere' may vary from as little as 100 km in young unstable regions to as much as 400 km beneath the older cratons. Oxburgh and Parmentier's model¹⁴ provides a mechanism for the growth of this tectosphere through diapiric uprise of refractory Mg-rich lithosphere from the subducting slab. In this context greenstone belts could be considered as merely the surface expression of this process, where more active diapirs have managed to penetrate thin, immature, sub-continental tectosphere. Once a substantial tectosphere has developed beneath a continent it would, for reasons of density, viscosity and temperature, offer more resistance to penetration by such diapirs. Perhaps it is no coincidence then that most greenstone belts occur in regions which were geologically very young at the time the belts formed.

Conclusions

This mechanism for the formation of Archaean ensialic greenstone belts may differ from that controlling the development of modern intra-oceanic marginal basins where, because of the more mature, cooler nature of the subducting lithosphere, the Benioff zone is steeply inclined. Toksöz and Bird³⁴ proposed that the down-dragging effect of the subducting slab induces convective circulation in the mantle wedge behind the volcanic arc, the broad upwardly convecting cell rupturing the lithosphere and causing back-arc spreading. Thus the effect may be different, but the strong link with subduction is present in both models. The original marginal basin model of Tarney *et al.*²² was based on the Mesozoic ensialic *rocas verdes* complex in southern Chile where the amount of back-arc extension was very limited. When all aspects of greenstone belts are considered we feel that, when due allowance is made for the different Archaean thermal

regime and mantle structure, a marginal basin analogue is the model best able to accommodate the observed features of greenstone belts.

An important corollary is that extreme geothermal gradients implied for the Archaean mantle on the basis of the high liquidus temperatures of komatiitic lavas⁷ are not necessarily correct. This is particularly important as far as eclogite stability in subduction zones is concerned because many Archaean crustal rocks have rare-earth element characteristics consistent with an origin through partial melting of eclogite³⁵⁻³⁷. Clearly also, caution must be exercised in deducing Archaean mantle compositions from high-magnesian komatiitic liquids; their compositions may merely reflect their local source regions.

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Efficient translation of prokaryotic mRNAs in a eukaryotic cell-free system requires addition of a cap structure

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In this and the accompanying paper we demonstrate that certain prokaryotic mRNAs, when modified at their 5'-termini with a cap structure, are translated in a eukaryotic cell-free protein synthesising system as efficiently as, or more efficiently than, eukaryotic mRNAs. Apparently, the prokaryotic mRNA contains all the information necessary for efficient recognition and initiation by eukaryotic translational components, except for the cap structure.

MANY eukaryotic mRNAs are modified at their 5'-termini with a cap structure consisting of 7-methylguanosine in 5'- to 5'-linkage with the first encoded base of an mRNA¹⁻³. *In vitro* translation experiments with various eukaryotic cell-free protein synthesising systems indicate that the presence of the cap moiety is required for efficient translation of these messages⁴⁻¹⁸. Although the degree of cap-dependent translation varies among different mRNAs and depends on the *in vitro* translation system used⁸ as well as on the particular conditions in which the translation reactions are carried out^{19,20}, the results clearly suggest an important physiological role for the cap structure in the expression of these mRNAs.

Various prokaryotic mRNAs (for example, RNA phage, T7 'early' RNA) have been translated in both mammalian and wheat-germ cell-free systems to produce accurate and functional protein products²¹⁻²⁷. However, in these experiments it was necessary to use relatively high concentrations of the prokaryotic mRNAs to obtain detectable synthesis of the protein products. The translational efficiencies of these prokaryotic mRNAs were significantly lower than the corresponding efficiencies exhibited by equivalent amounts of most eukaryotic mRNAs (as judged by the amino acid incorporation per mol of mRNA added to the translation reaction). The prokaryotic mRNAs were, in fact, translated at levels equivalent to those obtained with eukaryotic mRNAs lacking cap structures.

In this and the following article²⁸ we examine the translation of several well-defined prokaryotic mRNAs in a wheat-germ cell-free system and, in particular, the effect of the enzymatic addition of a cap moiety on the translational efficiencies of these mRNAs. Our results indicate that these messages can be translated at relatively high efficiency in the wheat-germ extract and, analogous to the situation described for many eukaryotic mRNAs, this efficient translation is absolutely dependent on modification of the prokaryotic transcript with the cap structure. For each case examined, the capped prokaryotic mRNA was

found to translate as efficiently as, or more efficiently than, the equivalent amount (per mol) of capped rabbit haemoglobin (Hb) mRNA. Moreover, the cap-dependent translation exhibited by these mRNAs has allowed: (1) detection of an endogenous capping activity present in wheat-germ extracts, (2) comparison of the relative translational efficiencies exhibited by an mRNA encoding the same gene when located at different positions within a transcript, (3) demonstration that an mRNA which atypically initiates with a 5'-pyrimidine triphosphate can be cap-modified and translated, and (4) comparison of the translational efficiencies exhibited by two mRNAs which encode the same gene product but differ in their 5'-non-coding regions.

Synthesis of the λ *cro* gene product

Transcription of phage λ DNA with purified *Escherichia coli* RNA polymerase yields four major, well characterised RNA transcripts (Fig. 1)²⁹⁻³¹. Two of these transcripts are relatively high molecular weight (MW) (>16S) polycistronic mRNAs which are known to code for a variety of phage functions. They initiate from the major early promoters of phage λ , p_L and p_R , and extend leftward and rightward, respectively, from the λ immunity region (Fig. 1). In addition, two smaller transcripts are synthesised (4S and 6S RNAs), but these do not code for viral proteins. If the *in vitro* transcription reaction is carried out in the presence of the transcription termination protein factor, *rho*, then the p_L and p_R initiated mRNAs are terminated early at sites t_L and t_R , respectively, resulting in the production of discrete lower MW monocistronic mRNAs. A 12S RNA which encodes the λN gene function is obtained from the left, and an 8S mRNA which encodes the λ *cro* gene product results from the right (Fig. 1)²⁹⁻³².

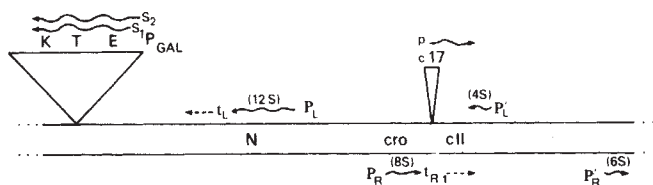


Fig. 1 Partial genetic map of bacteriophage λ showing the locations and relative sizes of the major RNA transcripts and some of the genes they encode. In this study λ^+ is actually $\lambda b2$, a derivative deleted for certain non-essential genes, thereby simplifying the *in vitro* RNA transcription pattern²⁹. *c17* represents a small DNA insertion which occurs within the intercistronic boundary between the genes *cro* and *cII* and creates a new RNA polymerase promoter site (*p*) for RNA transcription^{32,44,45}. The region of substitution of the entire galactose operon of *E. coli* (*gal* ETK) which occurs in the λ transducing phage, λ *pagal8*, is also indicated. All other designations are explained in the text.

RNA was prepared *in vitro* from a *rho* transcription reaction using λ DNA as template and then added to a wheat-germ cell-free translation system (Fig. 2). Addition of total RNA (that is, 4S, 6S, 8S and 12S RNA species) stimulated incorporation of ³⁵S-methionine into a single prominent polypeptide of ~7,500 daltons (Fig. 2-1). This protein was initially identified as the λ *cro* gene product by carrying out analogous translations on transcripts prepared identically from several well characterised λ mutants. The first, λ *x13*, carries a point mutation in p_R and is unable to initiate transcription of the 8S *cro* gene mRNA²⁹. The second, λ Δ *cro*, is deleted for the *cro* gene function. Although other RNAs are transcribed normally from the two mutants, these RNAs did not direct synthesis of the 7,500-dalton polypeptide (Fig. 2-2, 7). In contrast, translation of total RNA (all four species) prepared from a third mutant, λ *NN*⁻, which carries a double amber mutation in the *N* gene product (encoded by the 12S mRNA species)³³ did produce the 7,500-dalton polypeptide (Fig. 2-3). Thus, production of this polypeptide is apparently solely dependent on the presence of the 8S *cro* mRNA species. Identification was confirmed by preparing transcripts from a purified DNA restriction fragment which spanned

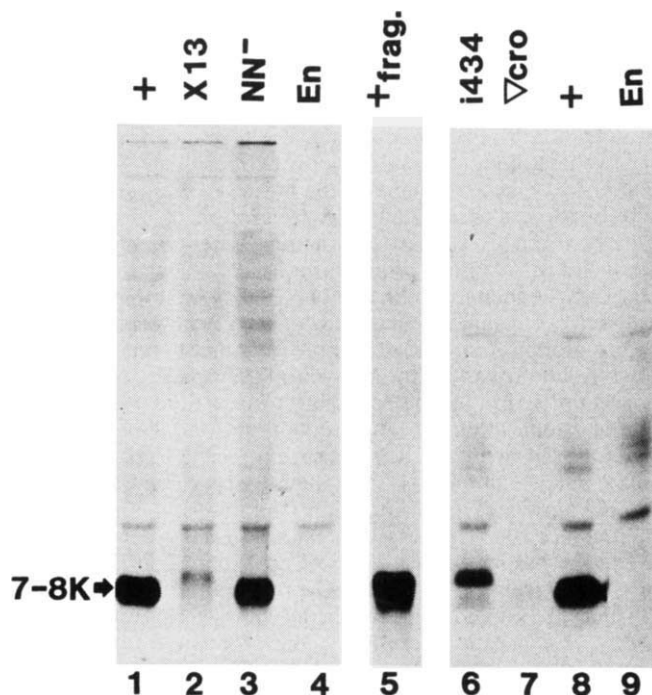


Fig. 2 Autoradiograph of a 12.5% SDS-polyacrylamide gel analysis of the ³⁵S-methionine-labelled cell-free products synthesised in wheat-germ extracts in response to RNA transcribed *in vitro* from the indicated λ phage DNAs (see text for description of the various λ derivatives). Wheat-germ translations were carried out (as described below) in 50- μ l reactions using the following components: (1) ~1.0 pmol of 8S *cro* mRNA contained in ~0.8 μ g total RNA prepared from λ^+ DNA; (2) ~0.8 μ g total RNA prepared from λ *x13* DNA; (3) 0.8 μ g total RNA prepared from λ *NN*⁻ DNA; (4) no added RNA (endogenous); (5) ~1.2 pmol *cro* mRNA contained in ~150 ng total RNA prepared from a purified λ DNA restriction fragment (see below and text); (6) ~0.4 μ g total RNA prepared from λ *imm434* DNA; (7) ~0.4 μ g total RNA prepared from a λ derivative which is deleted for the *cro* gene, λ *bio7-20nufL44cro* Δ 2; (8) same as (1); (9) same as (4). RNA transcriptions were carried out as previously described^{31,32} with the following modifications: reaction volume (0.5 ml), template DNA (2.5 pmol ml⁻¹), *E. coli* RNA polymerase (40 μ g ml⁻¹), purified *rho* factor (10 μ g ml⁻¹). RNA synthesis was monitored and quantitated by incorporating [α -³²P]ATP (specific activity 10 mCi mmol⁻¹) into a separate aliquot of the transcription reaction. Reactions were terminated by the addition of pancreatic DNase (25 μ g). After an additional 5 min incubation at 20 °C, the reaction mixture was made 0.1% in SDS, extracted with phenol, and precipitated twice with 2.5 volume of ethanol at -20 °C. The pellet was washed with 95% ethanol, dried and dissolved in H₂O. For certain reactions (1, 5 and 8) the relative yields of 4S, 6S, 8S and 12S RNA species were determined by analysing the aliquot of ³²P-labelled RNA on a 3.5% polyacrylamide slab gel containing 8.0 M urea^{32,33,51}. Wheat-germ cell-free translations were carried out essentially as described⁴⁶. Reaction mixtures (50 μ l) contained micrococcal nuclease-treated wheat-germ extract (10 μ l), SAM (5 μ M), ATP (1 mM), GTP (0.4 mM), magnesium acetate (2.0 mM), spermidine (600 μ M, free base), creatine phosphate (8 mM), creatine phosphokinase (8 μ g ml⁻¹; Sigma, 155 U per mg), KCl (85 mM), HEPES (25 mM, pH 7.6), amino acids minus methionine (25 μ M), dithiothreitol (2 mM), ³⁵S-methionine (10-15 μ Ci at 400-800 Ci mmol⁻¹, Amersham), and the indicated amounts of RNA. Protein synthesis was monitored by hot trichloroacetic acid precipitation as previously described⁴⁶. Preparation and running of the samples on the 12.5% SDS-polyacrylamide slab gel have also been described⁴⁶. After electrophoresis the gel was fluorographed for ~12 h at -70 °C (ref. 46). All values given represent final concentrations in the reaction mixture. The DNA restriction fragment used to prepare *cro* mRNA for translation reaction (5) was obtained from phage λ *r32*, a derivative which contains an IS2 insertion element within the *cro*-*cII* intercistronic boundary³². Restriction of this DNA with *Hind*III results in productions of a readily obtainable 1,600-base pair DNA fragment which spans the p_R -*cro* region (K. McKenney, unpublished data).

the *cro* cistron and contained only the p_R initiation site for RNA transcription. Translation of this RNA, which consists almost exclusively of the 8S *cro* mRNA, again results in production of the major 7,500-dalton polypeptide (Fig. 2-5).

Authentic *cro* protein has been obtained *in vivo* from λ -infected cells and is known to have a MW of 7,500 (ref. 34). Moreover, the nucleotide sequence of the region of the λ genome which encodes the 8S *cro* mRNA has been deter-

mined³⁵, as has the amino acid sequence of the purified protein³⁶. These data indicate that the 7,500-dalton cro polypeptide is a primary translation product and that the cro mRNA contains only one possible reading frame of codon triplets consistent with a protein of this size. Apparently, the 7,500-dalton protein being synthesised in the wheat-germ extract is the λ cro gene product. More recent studies (C. Queen, B.M.P. and M.R., unpublished results) indicate that translation of this same λ transcript in an *E. coli* S30 translation system results in the synthesis of the identical protein product.

RNA transcribed from the λ x13 phage DNA template directed synthesis of a minor polypeptide of slightly higher MW than the λ cro protein (Fig. 2-2). This product did not seem to result from translation of any of the major λ mRNA species, but rather was accounted for by the following observations. The λ x13 phage is a replication-defective mutant and requires a helper phage for its growth. The helper phage (in this case the hetero-immune phage λ imm434) is subsequently separated from λ x13 by CsCl gradient centrifugation, although some cross-contamination between the two phages usually occurs. The λ 434 phage has an entirely different and somewhat larger cro gene than λ^+ (ref. 37). Transcripts were prepared from purified λ 434 phage DNA and translated in the wheat-germ system (Fig. 2-6). Their translation resulted in the production of a single major polypeptide which had a gel mobility identical to the minor product observed from translation of the λ x13 mRNA (compare Fig. 2-2, 6). Apparently, the wheat-germ extract was able to translate the small amount of λ 434 cro mRNA which was transcribed with and contaminated the λ x13 mRNA prep-

aration. We emphasise that although the cro genes of λ^+ and λ 434 are functional analogues, the nucleotide sequences of these two genes show little, if any, homology even in those regions involved in ribosome recognition and initiation of protein synthesis (ref. 37 and V. Pirrotta, unpublished data). Thus, the two cro genes actually represent independent examples of prokaryotic sequences which are being recognised and translated by the wheat-germ components.

Synthesis of cro polypeptide is cap dependent

The cap structure is required for efficient translation of reovirus RNA^{4,38}. This was demonstrated by monitoring the relative translational efficiencies exhibited by reovirus RNA which had been prepared *in vitro* in the presence or absence of S-adenosylmethionine (SAM). SAM acts as a methyl donor in the capping reaction and is required for the formation of a functional cap structure². The viral RNA transcribed in the presence of SAM exhibited a significantly higher translational efficiency than RNA synthesised without added SAM. Moreover, it was observed that addition of S-adenosylhomocysteine (SAH) to the reovirus *in vitro* transcription system resulted in a sharp reduction in the ability to translate the reovirus RNA^{2,38}. SAH is an analogue of SAM which competitively inhibits formation of the cap structure on the reovirus RNA.

We have used similar criteria to determine the extent of cap-dependent translation of λ cro mRNA in the wheat-germ cell-free system. As before, cro mRNA was prepared *in vitro* as

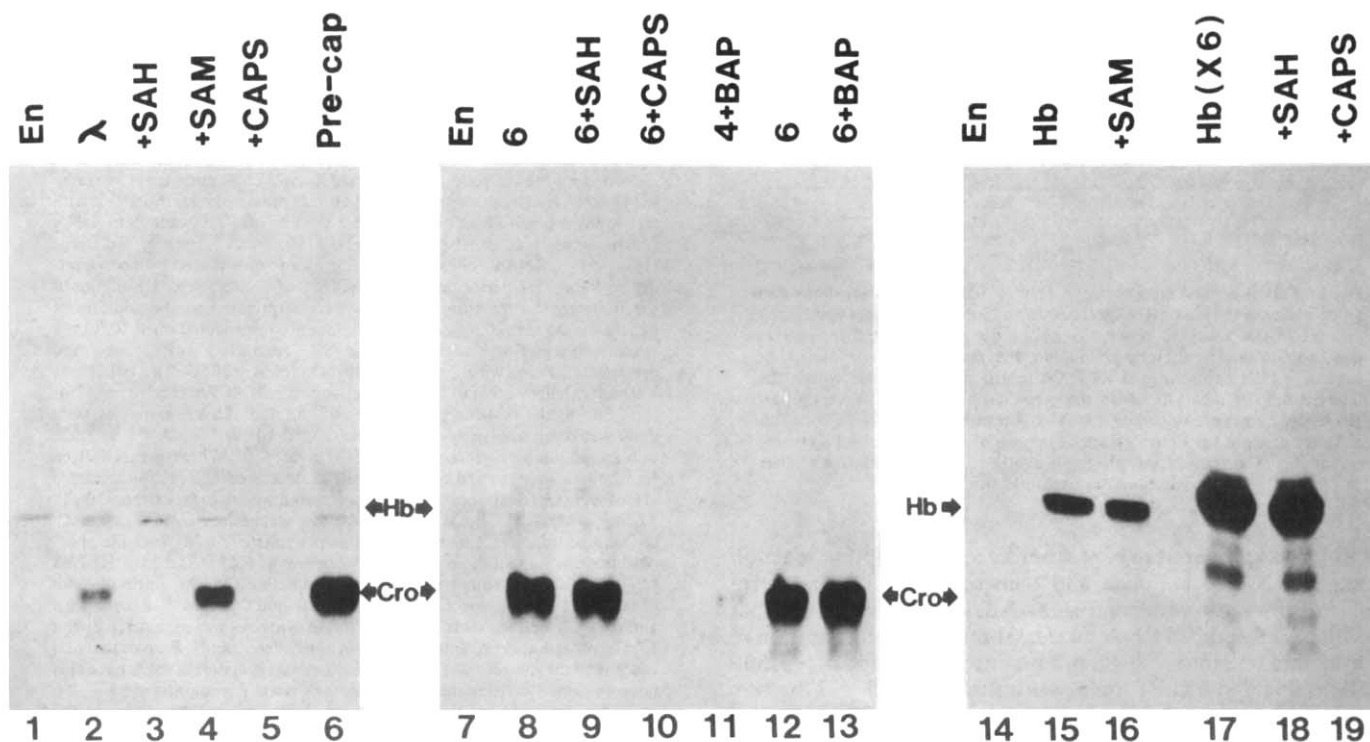


Fig. 3 Composite autoradiograph of an SDS-polyacrylamide gel analysis (as in Fig. 2) showing the effects of the indicated manipulations on the cell-free synthesis in wheat-germ extracts of the λ cro polypeptide (1-13) and haemoglobin (Hb) protein (14-19). λ^+ RNA was prepared *in vitro* (as described in Fig. 2) and identical aliquots of RNA (~0.5 pmol of 8S cro mRNA contained in ~0.4 μ g total RNA) were used for each translation reaction. Translations were carried out (as described in Fig. 2) except that S-adenosylmethionine was added only where indicated) in 50- μ l reactions using the following components: (1) no added RNA (endogenous); (2) λ RNA only; (3) λ RNA + S-adenosylhomocysteine (SAH, 500 μ M); (4) λ RNA + S-adenosylmethionine (SAM, 5 μ M); (5) λ RNA + m⁷GpppA (500 μ M); (6) λ RNA, which was initially cap-modified using the purified vaccinia capping enzymes (described below); (7) same as (1); (8) same as (6); (9) cap-modified λ RNA (as in 6) + SAH (500 μ M); (10) cap-modified λ RNA (as in 6) + m⁷GpppA (500 μ M); (11) λ RNA, which was initially treated with bacterial alkaline phosphatase (BAP, see below) + SAM (5 μ M); (12) cap-modified λ RNA (as in 6 and 8); (13) cap-modified λ RNA (as in 6), which was initially treated with BAP (as in 11); (14) same as (1) and (7); (15) Hb mRNA only (0.6 pmol); (16) Hb mRNA (0.6 pmol) + SAM (5 μ M); (17) Hb mRNA only (3.0 pmol); (18) Hb mRNA (3.0 pmol) + SAH (500 μ M); (19) Hb mRNA (3.0 pmol) + m⁷GpppA (500 μ M). RNA was cap-modified before translation using the purified vaccinia capping enzymes⁴⁰. Reaction mixtures (10-40 μ l) contained Tris-HCl (50 mM, pH 7.5), MgCl₂ (1 mM), GTP (1-2 mM), dithiothreitol (5 mM), SAM (0.1 mM), RNA (~0.4 μ g) and enzyme extract⁴⁰. After 20 min incubation at 37 °C, the reaction mixture was phenol extracted and precipitated twice with ethanol at -20 °C. The pellet was washed with 95% ethanol, dried, dissolved in H₂O and then used directly for translation. Bacterial alkaline phosphatase treatment of RNA was carried out in reaction mixtures (0.1 ml) containing Tris-HCl (5 mM, pH 0.8), MgCl₂ (5 mM), RNA (0.4-1.0 μ g) and BAP (1.0 μ g, Sigma). After 20 min incubation at 37 °C, EDTA (10 mM) was added and the reaction mixture was phenol extracted and precipitated twice with ethanol at -20 °C. The pellet was washed with 95% ethanol, dried, dissolved in H₂O and then used directly in the translation reaction.

a primary 8S mRNA and subsequently translated in a standard wheat-germ translation reaction (Fig. 3). In this case, however, the translation reactions were carried out in the presence and absence of SAM and SAH. Addition of SAM resulted in markedly enhanced synthesis of the cro polypeptide (about threefold, compare Fig. 3-2, 4). In contrast, SAH addition (Fig. 3-3) completely and selectively abolished the synthesis of cro. These data indicate that translation of cro in the wheat-germ extract depends on an endogenous SAM-mediated (SAH-inhibited) methylation reaction. Analogous to the situation described above for reovirus RNA translation, this methylase activity is presumably that which is associated with the formation of a functional cap structure.

The observed effects are specific for an mRNA which requires endogenous cap modification for its translation. Similar addition of either SAM or SAH to translation reactions containing purified Hb mRNA had no effect on the synthesis of Hb protein (Fig. 3-15-18). As Hb mRNA already contains a 5'-cap structure, its translation is independent of the effects of SAM and SAH on the wheat-germ methylation activity. This is also true for the products which result from translation of the endogenous wheat-germ mRNAs present in the various reactions (compare Fig. 3-1-4). These RNAs, like Hb, already contain a cap structure and thus serve as direct internal controls in the translation reactions. Thus, the specificity of the effects observed with cro mRNA indicates that an endogenous capping activity present in the wheat-germ extract can appropriately modify the 5'-triphosphate terminus of the prokaryotic transcript and lead to its enhanced ability to be translated in the cell-free system. The presence of an enzymatic capping activity in the wheat-germ extract was recently confirmed (B. Moss, personal communication).

We have also directly demonstrated cap-dependent translation of the cro mRNA using enzymatic capping activity from vaccinia virions^{39,40}. The enzymes were used to cap-modify the *in vitro* synthesised λ mRNA before translation in the wheat-germ system. The vaccinia enzymes add a cap structure to RNAs which contain either a 5'-di- or triphosphate terminus⁴⁰. Capping reactions were initially carried out with [α -³²P]GTP so as to monitor and quantitate the extent of cap modification of the λ transcripts (see Fig. 4 legend). Analysis of these enzymatically capped RNAs by polyacrylamide gel electrophoresis indicated that each of the λ transcripts had incorporated ³²P label (not shown). More importantly, enzymatic digestion of this RNA with T₁ RNase and subsequent two-dimensional fingerprint analysis of the products demonstrated that the ³²P label was specifically transferred to four oligonucleotides which had mobilities consistent with the cap derivatives of the known 5'-terminal oligonucleotides of each of the four λ transcripts (Fig. 4). The specific activity of the products indicated that ~60% of the cro mRNA was cap-modified; enzymatic addition of the cap structure to the other three transcripts ranged between 40% and 60%.

λ^+ RNA, prepared *in vitro* and capped with the vaccinia enzyme, was added to the wheat-germ cell-free translation system. Translation of this RNA resulted in a >10-fold increase in production of cro protein as compared with translation of an identical amount of untreated transcript (compare Fig. 3-6, 2). Thus, direct cap modification of ~60% of the input prokaryotic message resulted in a dramatic increase in its ability to be translated in this eukaryotic system. Moreover, these pre-capped transcripts now continued to direct the synthesis of cro protein even in the presence of SAH (Fig. 3-9), analogous to the translation of Hb mRNA (Fig. 3-18). Cap modification with the vaccinia enzymes has eliminated the need for endogenous cap formation during the translation reaction.

The requirement for the integrity of the 5'-triphosphate group on the cro mRNA primary transcript was also demonstrated. Removal of the terminal phosphate residues by initial treatment of the RNA with bacterial alkaline phosphatase (BAP), followed by addition of this RNA to a translation reaction (+SAM) abolished synthesis of the cro polypeptide (Fig. 3-11).

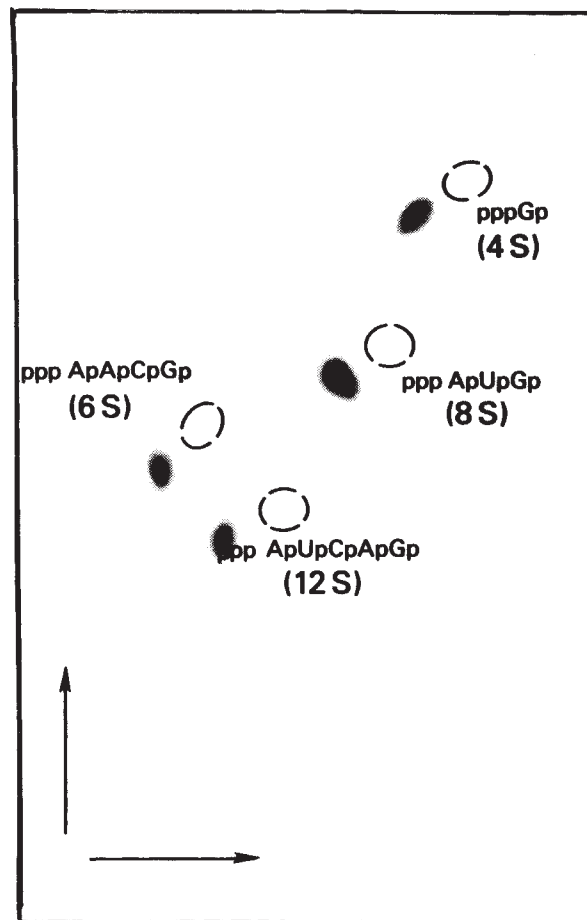


Fig. 4 Autoradiograph of a two-dimensional fingerprint (prepared as previously described)^{31,47,48} showing the 5'-terminal RNase T₁ oligonucleotide products resulting from cap modification of the four λ RNA transcripts. Unlabelled λ^+ RNA was prepared *in vitro* (as described in Fig. 2) and then cap-modified using the vaccinia enzymes, as described in Fig. 3 except that SAM was omitted from the reaction and [α -³²P]GTP (1-2 mCi, specific activity ~100 mCi μ mol⁻¹) was used to label specifically (by the cap-moiety) the 5'-triphosphate termini of the unlabelled transcripts. Horizontal dimension: electrophoresis on Cellologel in 8.0 M urea at pH 3.5. Vertical dimension: ascending thin-layer homochromatography on plates of DEAE-cellulose using homo-solvent B (refs 47, 48). The stippled circles show the approximate relative positions and nucleotide sequences of the unmodified 5'-terminal oligonucleotide products which are known to result from T₁ RNase digestion of the four λ transcripts (refs 49, 50, and unpublished data).

However, identical BAP treatment of λ RNA which had been pre-capped with the vaccinia enzymes had no effect on its ability to be translated (Fig. 3-13). Thus, the 5'-terminal triphosphate group was protected from phosphatase activity by the presence of the added cap structure.

Further evidence for the cap dependence of cro protein translation was obtained from experiments using m⁷G(5')pppA, a cap analogue which will inhibit the translation of capped mRNA^{14,20,41}. Cap analogues seem to have little effect on cap-independent translation²¹. Addition of m⁷G(5')pppA to the translation reactions abolished the synthesis of both the Hb and cro polypeptides (Fig. 3-5, 19), as well as the synthesis of endogenous wheat-germ proteins (that is, background; compare Fig. 3-1, 5). Identical results were obtained whether cap modification occurred during the translation reaction (in response to added SAM, Fig. 3-5) or before translation using the vaccinia enzymes (Fig. 3-10).

Experiments similar to those described for the λ cro gene product were also carried out with the λ imm434 cro gene. Essentially identical results were obtained (data not shown). Again, we emphasise that these two prokaryotic mRNAs are quite different in primary structure, but exhibit similar cap dependence. In contrast to the results obtained with cro protein,

we were unable to detect synthesis of the λ N protein, the gene product encoded by the 12S mRNA species. Although cap modification of this RNA was achieved using the vaccinia capping enzymes (Fig. 4), no obvious translation product was observed. Structural characterisation of the 5'-terminal region of the 12S RNA suggests that the translation start point for the N protein may be relatively far (>200 nucleotide residues) from the 5'-triphosphate end (ref. 41 and K. McKenney, unpublished data). In addition, the 5'-non-coding region of this transcript is

known to be processed *in vivo* into several discrete RNA fragments⁴³. Perhaps the length and/or structure of the 12S RNA leader region does not allow proper interaction between the 5'-terminal cap modification and the site for ribosome recognition and translation initiation of the N protein.

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Efficient cap-dependent translation of polycistronic prokaryotic mRNAs is restricted to the first gene in the operon

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Certain polycistronic prokaryotic mRNAs, when modified at their 5'-termini with a cap structure, are translated as efficiently as, or more efficiently than eukaryotic mRNAs in a eukaryotic cell-free protein synthesising system. However, in this case efficient cap-dependent translation is apparently restricted to the 5'-proximal coding sequence. Moreover, certain translational regulatory signals potentially used by these prokaryotic mRNAs to regulate their levels of expression seem to be recognised by the eukaryotic translational components. The evolutionary significance and practical implications of these results are discussed.

In the previous article¹ we demonstrated the cap-dependent translation of the monocistronic 8S λ cro mRNA in a wheat-germ cell-free protein synthesising system. These studies also allowed the detection of an endogenous capping activity present in the wheat-germ extract. Here, we extend these observations to other prokaryotic transcripts and, in particular, examine the cap-dependent translation of certain polycistronic mRNAs.

Cap dependence in a polycistronic operon

Transcription of λ DNA in the absence of the protein termination factor *rho* results in the production of two high molecular weight (MW) polycistronic transcripts which are derived from the elongation of the 8S and 12S mRNAs²⁻⁵ (see accompanying paper, Fig. 1). The *cro* gene now occurs at the 5'-end of a polycistronic transcript which is derived from the right operon of λ and encodes several other λ gene products. Translation of these high molecular weight mRNAs in the wheat-germ system again resulted in the synthesis of *cro* protein as the only major translation product (Fig. 1-1). Efficient translation of *cro* from the polycistronic message exhibited the identical dependence on cap modification to that observed for the 8S monocistronic mRNA (data not shown). The inability to detect protein synthesis from any of the cistrons located internally on the transcripts suggests that protein initiation sites positioned 'too far' from the cap structure may not be efficiently recognised by the eukaryotic translational components. Thus, it may be the relative proximity of the translation start site to the cap-modifiable 5'-triphosphate end of the transcript that is important for efficient *cro* protein translation. The following observations strongly support this contention.

The *cII* gene of λ comprises the second cistron of the p_R promoted major rightward operon (see accompanying paper, Fig. 1). This gene is located distal to the *cro* gene and separated from it by an intercistronic regulatory region of ~ 120 base pairs⁵. A well characterised λ mutant, $\lambda c17$, contains a small DNA insertion within the intercistronic boundary between *cro* and *cII*, immediately preceding the presumptive ribosome binding region of *cII* (refs 5–7). This insertion creates a new promoter site for RNA polymerase and results in the transcription and functional expression of the *cII* coding region⁷. Transcription from this *c17* promoter initiates atypically with a pyrimidine triphosphate (CTP) and this 5'-triphosphate end occurs only 18 residues from the presumptive AUG initiation codon for *cII* (Fig. 3)⁵. Thus, *in vitro* transcription of the $\lambda c17$ DNA template gives rise to one additional *c17* promoted transcript, which places the *cII* coding region in proximity to a new, cap-modifiable 5'-triphosphate end.

RNA transcripts were prepared *in vitro* using the $\lambda c17$ DNA template and subsequently translated in the wheat-germ cell-free system (Fig. 1). In contrast to the results obtained using the λ^+ DNA template, three major protein products were now obtained: the expected 7,500-dalton *cro* polypeptide and two additional proteins of $\sim 12,000$ and 14,000 daltons (Fig. 1–2). The single additional *c17* promoted transcript seemed to be responsible for the synthesis of both new polypeptides. This was confirmed when the same two protein products were obtained in

translation reactions carried out with RNA prepared from a purified DNA restriction fragment of $\lambda c17$ (data not shown). This DNA fragment contains the *c17* promoter and the entire *cII* coding region, but lacks the major p_L and p_R promoters of λ (ref. 5).

Translation of both new polypeptides, as well as the *cro* protein, was dependent on cap modification of the $\lambda c17$ mRNA. As with the translation of *cro* mRNA, synthesis of the two new polypeptides was markedly enhanced by S-adenosylmethionine (SAM) addition (Fig. 1–3) and sharply inhibited by S-adenosylhomocysteine (SAH) addition (Fig. 1–4) to the wheat-germ translation reaction. Moreover, cap modification before translation using the purified vaccinia enzymes again dramatically increased the production of both polypeptides (not shown).

Initial characterisation of the two new polypeptides was achieved by directing translation reactions with mRNA prepared from a derivative of $\lambda c17$ which carries an amber point mutation within the *cII* gene ($\lambda c17IIamber41$). Translation of the RNA surprisingly resulted in gel electrophoretic mobility shifts for both the 12,000 and 14,000 dalton polypeptides (Fig. 1–6). No change was detected for the *cro* polypeptide. As the amber mutation results from a single base-pair change⁹, both polypeptides must not only be derived from the *cII* coding region, but must also be translated in the same reading frame of codon triplets. Preliminary ³⁵S-methionine tryptic peptide mapping of these two *cII* proteins indicates that they do share

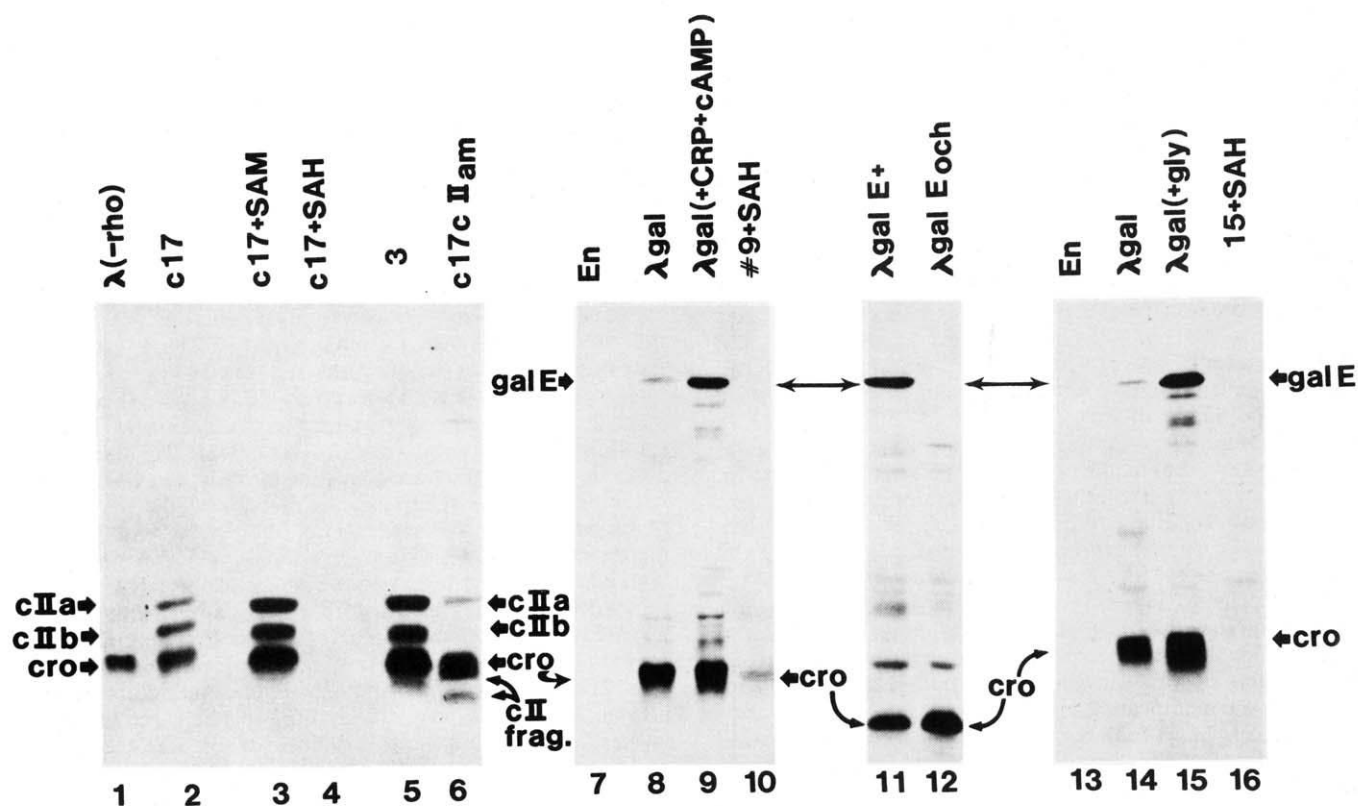


Fig. 1 Composite autoradiograph of a gel analysis (as described in Fig. 2 of the accompanying paper) showing the ³⁵S-labelled products synthesised in wheat-germ extracts in response to RNA transcribed *in vitro* (see accompanying paper, Fig. 2) from various λ phage DNAs (see text for description of each λ derivative). Wheat-germ cell-free translations were carried out (as described in Fig. 3 of the accompanying paper) in 50- μ l reactions using the following components: (1) λ^+ RNA (as in Fig. 3 of the accompanying paper except that *rho* factor was omitted from the transcription reaction); (2) $\lambda c17$ RNA (containing ~ 0.6 pmol 8S *cro* mRNA in 0.5 μ g total RNA); (3) $\lambda c17$ RNA (as in 2) + SAM (5 μ M); (4) $\lambda c17$ RNA (as in 2) + SAH (500 μ M); (5) same as (3); (6) $\lambda c17cIIamber$ RNA (~ 0.4 μ g) + SAM (5 μ M); (7) no added RNA (endogenous); (8) $\lambda gal8$ RNA, prepared in the absence of *rho* factor; (9) $\lambda gal8$ RNA, prepared as in (8) except that CRP (40 μ g ml⁻¹) and cyclic AMP (0.3 mM) were added to the transcription reaction; (10) same RNA as in (9) + SAM (5 μ M); (11) same as (9); (12) $\lambda galochreb4$ RNA, prepared with CRP (40 μ g ml⁻¹) and cyclic AMP (0.3 mM) added to the transcription reaction; (13) same as (7); (14) same as (8); (15) $\lambda gal8$ RNA, prepared as in (8) and (14) except that glycerol (20%) was added to the transcription reaction; (16) same RNA as in (15) + SAH (500 μ M). All values given are final concentrations in the reaction mix. The two presumptive *cII* fragments indicated in (5) and (6) have not been shown to be derived from the *cIIa* and *cIIb* polypeptides. This conclusion is the simplest interpretation of the gel pattern and is consistent with the position of the *cIIamber41* mutation. The differences in relative intensity observed for the two *cII* fragments presumably result from the corresponding changes which occur in their respective methionine content. The minor band which remains at the *cIIa* position is an endogenous product (compare with Fig. 2-4 or 3-1 of the accompanying paper). The amounts of *gal E* specific mRNA added to translation reactions (8)–(16) were not determined; however, each translation reaction was directed with an identical aliquot of RNA obtained from each of the $\lambda gal8$ transcription reactions. Furthermore, these aliquots of RNA were estimated to approximate to those used in translation reactions 1–6.

common peptides and that the smaller protein consists mainly of a subset of the tryptic products contained in the larger protein (unpublished results). Moreover, the amber mutation seems to have resulted in a similar reduction in MW for each polypeptide (Fig. 1 see legend). If this is correct, then both the cII polypeptides must have the same (or nearly identical) carboxy termini and differ only (or predominantly) at their amino-terminal ends (by ~20–25 amino acid residues, Fig. 3).

An authentic 14,000 MW protein, which is derived from the cII coding region, has been identified from λ -infected cells¹⁰. The DNA sequence of the cII coding region predicts a protein of this MW⁹. Thus, the larger of the two cII proteins (cIIa, Fig. 1) synthesised in wheat-germ in response to the capped c17 transcript is likely to be this protein. The smaller 12,000-dalton polypeptide (cIIb) probably initiates translation 20–25 codon positions to the right of this site. The apparent size reduction resulting from chain termination observed for the two proteins would then be entirely consistent with the known nucleotide position of the amber mutation in the cII gene. Furthermore, a second AUG codon does occur at amino acid position 23 of the cIIa protein (position 84–87 in the c17 mRNA, Fig. 3). This AUG might serve as the initiator codon of cIIb. Ribosome binding studies support this contention (M.R. and B.M.P., in preparation). In separate reactions, translation components from either wheat-germ or *Escherichia coli* were used to form stable translation initiation complexes with the purified c17-promoted transcript. Both the 60S prokaryotic ribosome and the 70S eukaryotic ribosome were found to bind specifically and protect (from ribonuclease digestion) two separate and identical regions of this RNA. Each protected site spanned about 30–40 nucleotide residues and centred around one of the appropriate AUG codons. It is not known whether these two overlapping sites for protein synthesis have any functional significance for the bacteriophage. However, recent studies (C. Queen, B.M.P. and M.R.) indicate that two proteins of identical electrophoretic mobilities to those made in wheat-germ can be synthesised in an *E. coli* S30 translation system using the same *in vitro* prepared λ transcripts. Thus, the wheat-germ translational components may be recognising a translational regulatory mechanism used by λ to produce two different polypeptides from the same coding region.

Regardless of the possible functional implications of the two overlapping cII proteins, their translational in the wheat-germ system required cap modification of the c17-initiated mRNA. As we described earlier, the sequences encoding the cII regulatory region also occur on the p_R -initiated polycistronic transcript which was synthesised from the λ^+ DNA template. In this case, cII is the second cistron of the operon, located ~330 nucleotide residues away from the cap-modified 5'-end. Translation of the p_R -promoted mRNA did not result in detectable synthesis of either cII protein (Fig. 1-1). Only on introduction of a new transcription start site (through the c17 promoter), which placed a cap-modifiable 5'-triphosphate terminus in the proximity of the cII coding region, was efficient translation of cII obtained. Moreover, cap modification of the initiating pyrimidine nucleotide, CTP, of the c17 mRNA resulted in efficient translation from two different sites, respectively, 18 and 84 residues from the cap structure. The larger protein (initiated closer to the cap) was always obtained in somewhat higher yield (~60%) than the smaller cII protein (~40%, normalised for methionine content). We do not know whether the two start sites represent functionally independent regions for translation initiation or whether their function is related. The mechanism which leads to the cap-dependent synthesis of the two cII proteins is being studied.

Cap-dependent *gal* E translation

The *gal* operon of *E. coli* consists of three structural genes, *E* (epimerase), *T* (transferase) and *K* (kinase), which are transcribed into a single polycistronic mRNA from a promoter region which has been well characterised^{11–13} (see accompanying paper, Fig. 1). *In vitro*, transcription of *gal* DNA can occur

from either of two distinct sites which are known to be 5 base pairs apart within the *gal* regulatory region (start sites *S*₁ and *S*₂, Fig. 3)¹⁴. Transcription from start site *S*₁ requires the presence of cyclic AMP receptor protein (CRP) and cyclic AMP in the transcription reaction¹⁴. In these conditions, no transcription initiates from start site *S*₂. In the absence of CRP and cyclic AMP, *S*₁ transcription is abolished and only low-level *S*₂ transcription occurs (~20-fold lower than the CRP-cyclic AMP-dependent *S*₁ transcription). However, CRP-cyclic AMP-independent *S*₂ transcription can be stimulated (~8–10-fold) by the addition of glycerol to the *in vitro* reaction¹⁵. Thus, using the appropriate *in vitro* transcription conditions, two distinct transcripts of the *gal* operon can be obtained which differ only in that one (CRP-cyclic AMP independent) has five additional nucleotide residues at its 5'-end compared with the other (CRP-cyclic AMP-dependent transcript) (Fig. 3).

RNA was prepared *in vitro* using DNA obtained from a λ transducing phage, λ_{pgal8} , which carries the entire *gal* operon within its genome (see accompanying paper, Fig. 1). These transcripts consist of the various λ mRNAs (described earlier), as well as the additional *gal*-derived polycistronic mRNA. Transcriptions were initially carried out either in the presence or absence of CRP-cyclic AMP and this RNA was then translated in the wheat-germ cell-free system (+SAM, as described in Fig. 1). RNA, prepared in the presence of CRP-cyclic AMP, directed synthesis of a new major protein product (~38,000 daltons, Fig. 1-9). Only minor amounts of this same protein were produced in a translation reaction carried out identically on RNA made in the absence of CRP-cyclic AMP (Fig. 1-8). Transcription of the *gal* operon, specifically induced by CRP-cyclic AMP, resulted in the corresponding translation of the 38,000-dalton protein. Cro protein was also made in these reactions (in response to the λ_{pR} -initiated mRNA); however, its synthesis was not affected by the presence or absence of CRP-cyclic AMP (compare Fig. 1-8 and 1-9). The CRP-cyclic AMP-dependent protein product is presumably *gal* epimerase (*E*), the 38,000-dalton protein encoded by the first cistron of the *gal* operon. Identification of the *gal* *E* product was confirmed by the translation of transcripts prepared from a mutant of λ_{pgal8} , which carries an ochre, chain-terminating mutation early within the *E* gene (S. Adhya, unpublished). These transcripts specifically failed to direct synthesis of the 38,000-dalton protein, whereas cro protein was produced normally (Fig. 1-12).

Analogous to the results obtained with both *cro* and cII, *gal* *E* translation in the wheat-germ system was also completely dependent on cap modification of the *S*₁-initiated *gal* transcript. Addition of SAH to the translation reaction abolished synthesis of the *gal* *E* protein (as well as cro protein being synthesised in the same reaction, Fig. 1-10). In addition, *gal* *E* synthesis was markedly enhanced by cap modification of the RNA with the purified vaccinia enzymes before its translation. Although the *gal* mRNA encodes three gene products (*E*, *T* and *K*), again only the 5'-proximal *E* gene seemed to be translated efficiently. The AUG initiation codon for *gal* epimerase occurs only 27 residues from the 5'-cap-modified triphosphate end of the *S*₁-initiated *gal* transcript¹⁵. It is difficult to compare the relative cap-dependent translation efficiencies of *gal* *E* and *cro* as the number of methionine residues in epimerase is not known. However, the *gal* promoter, even when stimulated 20-fold by CRP-cyclic AMP, is still less efficient *in vitro* than the λ_{pR} promoter. Thus, the apparently weaker translational response observed for *gal* *E* (compared to *cro*) results, at least in part, from the fact that there is less *gal* RNA produced in these transcription reactions than there is *cro* mRNA.

Several minor protein bands also seem to be synthesised in the wheat-germ extract in response to the CRP-cyclic AMP dependent *gal* transcript (Fig. 1-9). The appearance of these products was also sensitive both to the effects of SAH (Fig. 1-10) and to the introduction of the *gal* *E* ochre chain-terminating mutation (Fig. 1-12). These minor products probably result from premature termination of either *gal* *E* transcripts or translation products and were not further characterised.

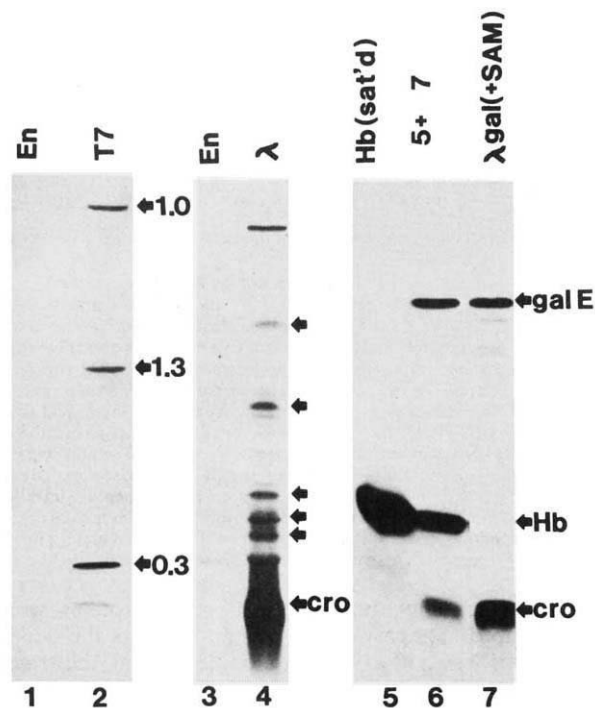


Fig. 2 Composite autoradiograph of a gel analysis showing the ^{35}S -methionine-labelled products synthesised in wheat-germ extracts in response to RNA transcribed *in vitro* from the indicated phage DNAs (1–4) and a competition experiment comparing the relative translational efficiencies of *cro*, *gal E* and Hb mRNA (5)–(7), see below and text for details. Translation reactions 1–4 were carried out (as in Fig. 1) using the following components: (1) no added RNA (endogenous); (2) T7 early RNA (2.0 μg total RNA containing ~ 1.0 pmol of polycistronic T7 mRNA); (3) same as (1); (4) λ^+ RNA, transcribed in the absence of *rho* factor (2.0 μg total RNA containing ~ 1.0 pmol of the *p_R*-initiated polycistronic *cro* mRNA). Translation reactions (5)–(7) were carried out (+5 μM SAM) in reaction mixtures (50 μl) containing only 50% of the wheat-germ extract (5 μl) as was used in all previous reactions (see Fig. 2 of the accompanying paper). This 'diluted' cell-free system was found to be saturated for translation of 3.0 pmol of Hb mRNA. Translations were carried out with the following components: (5) Hb mRNA only (3.0 pmol); (6) Hb mRNA (3.0 pmol) + $\lambda\text{gal}18$ RNA (containing ~ 0.5 pmol of *cro* mRNA and even less (but an undetermined amount) of *gal E* mRNA (see text)); (7) $\lambda\text{gal}18$ RNA only (as in (6)). RNA was transcribed from T7 DNA as previously described²⁹, with the following modifications: reaction volume (1.0 ml); T7 DNA ($100 \mu\text{g ml}^{-1}$); RNA polymerase ($50 \mu\text{g ml}^{-1}$). The T7 RNA directed translation products were identified by comparing their electrophoretic mobilities with those T7 products identified by Anderson *et al.*¹⁷. Apparent λ translation products ($\rightarrow$), other than *cro* polypeptide, were not identified. Gel analyses of translation reactions (1)–(4) were autoradiographed for ~ 24 h (twice the exposure time of all other gel analyses).

gal E from another mRNA

Experiments similar to those described above were also carried out with RNA transcribed in the presence of glycerol using the $\lambda\text{gal}8$ DNA template. Glycerol affects the level of RNA synthesis occurring from both the λ and *gal* promoters¹⁵. CRP-cyclic AMP-independent *gal* transcription (from start site *S*₂, Fig. 3) is increased 8–10-fold, whereas transcription of the λp_R operon is enhanced only twofold¹⁵.

RNA was purified from a glycerol-stimulated transcription reaction and translated in the standard wheat-germ cell-free system. Again, two major protein products were synthesised: the 7,500-dalton *cro* protein and the 38,000-dalton *gal E* protein (Fig. 1–15). The translation system seems to have responded to the glycerol-enhanced levels of both *cro* and *gal* RNA (compare with Fig. 1–14). Using the same criteria as before, these transcripts were also shown to require cap modification for their translation in the wheat-germ extract (see, for example, Fig. 1–16).

gal mRNA stimulated with glycerol initiates at start site *S*₂ and has a 5'-non-coding region 32 nucleotides long¹⁴. This places the cap structure 5 nucleotides farther from the presumptive *gal E* initiation site than occurs on the CRP-cyclic AMP-dependent, *S*₁-initiated mRNA. Although both

gal mRNAs are dependent on cap modification for their efficient translation in the wheat-germ system, these two messages exhibited different translation efficiencies. Approximately twice the amount of *gal* mRNA is made in the CRP-cyclic AMP-dependent transcription reaction compared with the glycerol-stimulated reaction¹⁵. However, the more abundant CRP-cyclic AMP-dependent *S*₁ mRNA directed synthesis of less ($\sim 50\%$) *gal E* protein than did the glycerol-induced *S*₂ mRNA (compare Fig. 1–9, 15). Assuming that cap modification occurs to similar extents on the two different mRNAs, then the *S*₂-promoted mRNA is being translated 4–5-fold more efficiently than the *S*₁-promoted mRNA.

It was surprising that the *gal* mRNA was translated more efficiently when the cap moiety occurred 5 nucleotides farther from the translation start point. Presumably, the translational differences exhibited by the two *gal* mRNAs reflect the corresponding differences in their 5'-terminal structures (Fig. 3). The primary structural differences may affect the higher order structures (secondary and tertiary) exhibited by the non-coding leader regions of these two RNAs. Thus, the overall structure of the regions involved in recognition and initiation of *gal E* translation may be different in the two *gal* messages and thereby account for the differences in their translation efficiencies. As the translation system is derived from wheat-germ and requires cap modification of the *gal* mRNAs, the observed effects may be specific for translation in this particular system and therefore somewhat artefactual.

Alternatively, it is possible that the wheat-germ system is recognising and responding to a translational regulatory mechanism normally used to regulate *gal* enzyme levels in bacteria. The *gal* operon is known to be coordinately expressed when cells are metabolising galactose¹¹. In the absence of galactose, however, *gal E* expression is constitutively required for bacterial cell-wall synthesis¹¹. It has been postulated that the dual promoter system of *gal* regulates these different requirements for *gal* enzyme levels. Recent experiments (S. Adhya, personal communication) further indicate that, *in vivo* during CRP-cyclic AMP-independent *gal* expression, *gal E* activity is fourfold higher (whereas *gal K* activity is lower) than during CRP-cyclic AMP-dependent *gal* expression. If (as *in vitro*) the CRP-cyclic AMP-independent promoter (from *S*₂) is less efficient at *gal* transcription than the CRP-cyclic AMP-dependent promoter (from *S*₁), then perhaps the higher levels of *gal E* activity observed in the absence of CRP-cyclic AMP can be accounted for by more efficient translation of the *S*₂-initiated mRNA. If this is the case, then the wheat-germ components may not only be efficiently translating the cap-modified prokaryotic mRNA, but may also be responding to other translational regulatory signals encoded within these transcripts.

Efficiency of capped and uncapped prokaryotic mRNAs

Identical amounts (~ 0.5 pmol) of *cro* mRNA were added to each of the translation reactions depicted in Fig. 3 of the accompanying paper. However, the amounts of *cro* protein synthesised in each of these reactions differed, due to the different relative extents to which the *cro* mRNA had been cap-modified in the various reactions. Approximately 60% of the *cro* mRNA (0.3 pmol) was shown to be cap-modified using the vaccinia enzyme. Translation of this RNA resulted in a >10 -fold enhancement in *cro* protein synthesis, as compared with direct translation of the λ transcript (see accompanying paper¹, Fig. 3–2, Fig. 3–6). If the translational activity exhibited by the *cro* mRNA is proportional to the number of functionally capped transcripts, then the amount of *cro* product obtained from direct translation of the λ RNA represents cap modification of $<6\%$ of the input RNA (that is, <0.03 pmol). Addition of SAM to the translation system was shown to stimulate *cro* translation about threefold. Thus, the wheat-germ extract seems to be capable (in the presence of SAM) of cap modifying about 15–20% of the input *cro* mRNA (that is, ~ 0.1 pmol) and translating it with relatively high efficiency.

translation of at least some prokaryotic mRNAs. It has also been suggested that the 5'-non-coding region of the eukaryotic mRNA may contain a similar type of recognition signal which correspondingly interacts with the 3'-end of the eukaryotic 18S rRNA component²⁵. However, the heterogeneity in size and nucleotide sequence of the 5'-non-coding region of the eukaryotic mRNAs suggest that, except for the AUG initiation codon, it is difficult to find a conserved region which exhibits the necessary complementarity^{26,27}.

The prokaryotic mRNAs used in our studies also exhibit little complementarity (except for the AUG initiation codon) to the 3'-end of the eukaryotic 18S rRNA. The *λcro* and *cII* mRNAs, for example, can form only 3 A · U base pairs with the eukaryotic rRNA component. In contrast, these same mRNAs exhibit relatively extensive complementarities to the bacterial 16S rRNA component (Fig. 3). The ability of the prokaryotic mRNAs to be translated so efficiently by the wheat-germ system suggests that the necessity for the complementarity feature has been markedly diminished in the eukaryotic translation system. Although it is possible that an alternative recognition signal(s) has evolved in the eukaryotic mRNA, this signal should not be present in the prokaryotic message. As cap modification is the

only alteration required for efficient translation of the prokaryotic mRNA, perhaps the cap moiety now fulfills this recognition function.

The ability of the cap-modified prokaryotic message to compete efficiently with even saturating amounts of a eukaryotic mRNA (Fig. 2-6) suggests the possibility of obtaining high-level, functional expression of prokaryotic genes on their introduction into eukaryotic cells. Of course, appropriate transcriptional and post-transcriptional modification of the prokaryotic sequences would be required. Furthermore, it may be possible to increase the level of synthesis of essentially any gene product within a eukaryotic cell. Those prokaryotic sequences which contain highly efficient recognition signals for eukaryotic translational components (for example, 5'-non-coding region of the *λcro* gene) could be fused (using available genetic engineering techniques) to eukaryotic sequences encoding protein structural information. Experiments of this nature are under way.

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letters

A kinematic model for SS433

THE bizarre object SS433, first noted because of its H α emission¹ and most recently because it is the optical counterpart of a variable radio and X-ray source²⁻⁴, exhibits an extraordinary optical spectrum⁵. Three sets of emission lines of hydrogen and helium appear: one is near zero radial velocity (less than 180 km s⁻¹), and the other two show large and variable shifts to the blue and red. These shifts are almost unquestionably due to the Doppler effect, as multiple lines with constant $z \equiv \Delta\lambda/\lambda$ have been identified in both the red and blue systems⁶⁻⁸. The observed range of shifts^{5,8} corresponds to velocities from 0 to -30,000 km s⁻¹ in the blueshift system, and 11,000 to 48,000 km s⁻¹ in the redshift system, with the two sets of lines moving $\sim 180^\circ$ out of phase with each other. At present the red- and blueshift variations seem periodic, with a period of about 164 d, based on the observation of parts of three cycles^{7,8}.

The emitting regions giving rise to the variable wavelength systems cannot be objects in mutual orbital revolution, for with a 164-d period and velocities of 0.15 *c*, keplerian orbits would have light-crossing times of the order of weeks, and the blue- and red-shifted emission lines could not continue to have simultaneous oppositions. The emission, therefore, must come from regions relatively close to a central object. A lower limit on the

distance to SS433, based on interstellar line strengths and velocities⁹, is about 3.5 kpc. The low velocity of the zero-redshift system suggests that the object is probably galactic, although certain *ad hoc* extragalactic hypotheses cannot be ruled out.

Milgrom⁹, independently of the present work, has also noted that the variations in redshift could be periodic and sinusoidal, and he considered several possible models to account for the variable velocity systems, including what we believe to be the correct one. In our judgement, Milgrom showed remarkable insight in interpreting the few data⁵ then available to him. Now the much expanded observations⁸ are sufficient to narrow the possibilities substantially.

We suggest here that the radiation with variable Doppler shift is emitted by hot matter ejected by the central object at high but nearly constant velocity in oppositely directed narrow streams, possibly along a magnetic axis. Rotation of the beam axis provides the observed radial velocity variations. This model is thus a modification of that proposed for SS433 by Fabian and Rees¹⁰, where the beam velocity is modulated to create these variations. In our interpretation for SS433, as the matter reaches a critical distance from the central object, it cools sufficiently to recombine and give rise to the observed emission. The stationary system of lines may originate from low velocity gas in the vicinity of the object or possibly from matter ejected at an earlier

time and later re-excited. The radiating matter could also be infalling along a collimated axis, although the source of such matter then requires special explanation. Conversely, the matter can be ejected (or infalling) isotropically from (or onto) the object, and be illuminated by two ionising radiation beams from the centre. The problem of accounting for the copious source of material in the latter case is far from trivial. We are concerned here not with a specific mechanism, but rather with a kinematic model that accounts for the current observations; it is very similar to model 3 suggested earlier by Milgrom⁹. Our model includes specific predictions regarding the behaviour of the variable velocity components during the as yet unobserved portions of the 164-d period. In particular, the behaviour in the summer of 1979 is predicted to show correlated variations with much lower amplitude than previously observed. This asymmetric behaviour, if observed, will provide some guide to the range of astrophysical mechanisms involved, and also yield direct observational evidence that the system period is actually 164 d, and not an integer multiple thereof.

We assume that the beam axis rotates with angular velocity $\omega = 2\pi/164$ d. Although the cause of this rotation is unimportant to our simple kinematic description, note that the extremely high ratio of total system luminosity to the rotational energy of a compact star with a 164-d period implies that simple stellar rotation locked to the beams may not be the basic underlying system clock; precession is a possible alternative¹¹. Let the line-of-sight lie along a unit vector $\hat{n}$, inclined at angle i to the beam rotation axis. As the beam axis rotates, the jets of flowing matter, orientated at angle θ to the rotation axis, describe a conical motion about this axis, with period 164 d. Let the matter stream in opposite directions at speed v (in units where $c = 1$), and let $\mathbf{V}$ be the velocity vector of the stream in the same hemisphere as $\hat{n}$. The direction cosine, l , of the angle between $\mathbf{V}$ and $\hat{n}$ is

$$l = \frac{\mathbf{V}}{v} \cdot \hat{n} = \sin i \sin \theta \cos \omega t + \cos i \cos \theta$$

$$\equiv a \cos \omega t + b \quad (1)$$

The corresponding direction cosine, l' , of the angle between the line of sight and the opposite stream is

$$l' = -l = -a \cos \omega t - b \quad (2)$$

Note that

$$a + b = \cos |\theta - i|$$

$$b - a = \cos (\theta + i) \quad (3)$$

and

The Doppler shift is simply

$$1 + z = \gamma(1 + lv) \quad (4)$$

where l must be the direction cosine in the frame of the observer, and $\gamma = (1 - v^2)^{-1/2}$. The observed Doppler shifts of the streams with direction cosines l and l' are thus:

$$(1 + z) = \gamma(1 + va \cos \omega t + vb)$$

$$(1 + z') = \gamma(1 - va \cos \omega t - vb) \quad (5)$$

Thus va and vb are determined conveniently by observations of the ratio of $(1 + z)$ to $(1 + z')$ at any two phases and v follows from equations (5).

Figure 1 shows the measured Doppler shifts of SS433 obtained on 55 nights between June 1978 and April 1979, and folded with a 164-d period. These data were obtained from refs 5, 6 and 8, as well as re-interpretation of data^{4,12} where wavelengths of the moving features were tabulated, but not previously interpreted as the Doppler effect. Note that a con-

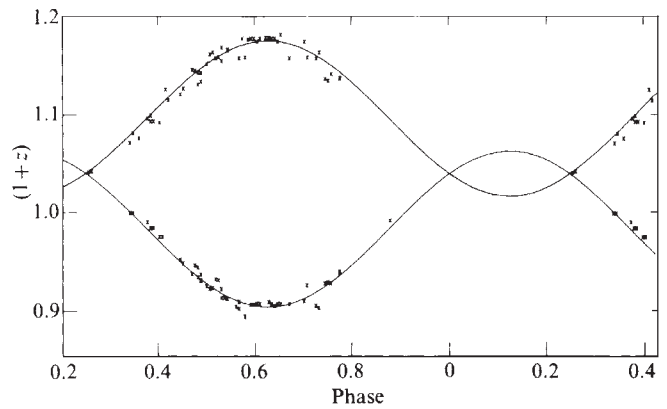


Fig. 1 Red- and blueshifts of SS433, measured on 55 nights during 1978-79, from sources cited in the text. The data have been folded with a 164-d period, and slightly more than one cycle is shown for clarity. The phase convention is arbitrary; as shown here, phase zero occurs on approximately 17 January 1979. The solid line is the prediction of the theoretical model described in the text, for beam velocity $v = 0.270$.

tiguous 35% portion of the entire cycle has never been observed. We take the point of largest amplitude of the radial velocity curves to correspond to the phase where $\cos \omega t = 1$ (a choice of $\cos \omega t = -1$ is simply a rotation of coordinates through 180° , and does not change the solution). Then the phase $\cos \omega t = 0$ occurs at one-fourth cycle on either side of the maximum, and is a convenient second measurement point in our data. From Fig. 1 we find that the amplitudes of the radial velocity curves at the two relevant phases are: for $\cos \omega t = 1$, $(1 + z) = 1.175 \pm 0.005$ and $(1 + z') = 0.903 \pm 0.005$; and for $\cos \omega t = 0$, $(1 + z) = 1.095 \pm 0.007$, $(1 + z') = 0.982 \pm 0.005$. We thus derive $va = 0.0765$, $vb = 0.0544$ and $v = 0.270$.

We cannot rule out a zero-point offset, Δ , in the observed velocities due either to a cosmological (Hubble) redshift or gravitational redshift. Note, however, that the solution for va and vb , as described above, is independent of v . Because each depends on ratios $(1 + z)/(1 + z')$, va and vb are also very insensitive to choices of moderate values of Δ . Thus va and vb are well determined; a choice of Δ determines v , and vice versa, but a range of values of Δ and v are compatible with the data. However, a value of $v > 0.270$ requires a positive value of Δ , which if cosmological, requires that the system of stationary lines arises from gas with a velocity $c\Delta$ in our direction, accidentally cancelling Δ , and which if gravitational, poses severe difficulties in finding astrophysical mechanisms for the emission. To minimise such astrophysical problems and assumptions, we adopt $\Delta = 0$ and $v = 0.270$. Then equations (5) generate the variation of $(1 + z)$ and $(1 + z')$ with phase, shown as smooth curves in Fig. 1.

We find the angles i and θ from equations (3), but cannot tell which is which, that is $(i, \theta) = (78^\circ, 17^\circ)$ or $(17^\circ, 78^\circ)$. The fact that the beams are found to lie at least 61° from the line of sight at all phases implies that any 164-d phase dependence of the moving system linewidths, caused by viewing the angular spread of the beam from different orientations, should be less than 13%, too small to be observable in our data sample.

Equations (5) also describe the situation⁹ in which a rapidly revolving disk or ring of gas in a plane is illuminated by two pencil beams of ionising radiation from the central object, with a 164-d period of rotation. In this case, however, because the velocity variations are always confined to this plane, $z = z'$ at $\cos \omega t = 0$, contrary to the observations of Fig. 1. Hence these ring or disk models must be ruled out.

In conclusion, Fig. 1 predicts that on or about 1 July 1979 the two moving emission line systems in SS433 will briefly merge.

into one, similar to a previously reported episode⁶, and that for the following 40 days the lines will separate again, but by an amount much less than previously observed.

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Is Cassiopeia A a black hole?

It is rather curious that no European astronomer observed the supernova which occurred in our Galaxy in about 1668 and left the remnant Cassiopeia A. However, if the absolute magnitude of the supernova is $M_V = -19$ mag, the interstellar absorption is $A_V = 4.3$ mag (ref. 1) and the distance is 2.8 kpc. Then its maximum visual magnitude should have been about -2.5 mag. Even ~ 100 d after the event, the supernova should have been as bright as any star in the Cassiopeia constellation. The fact that no such object was recorded suggests that the optical luminosity of the supernova may have been several orders of magnitude lower than the usual value. This note argues that the low luminosity of Cas A may be an indication that the product of the explosion is a black hole.

Recently Chevalier² hypothesised that Cas A was a 'dwarf' supernova born in the explosion of a fairly massive compact star whose photosphere extended not more than 10^{12} – 10^{13} cm and which had lost its hydrogen-rich envelope during its evolution. The fact that pre-type I-supernovae are also compact objects which lost their hydrogen envelopes during their evolution³ conflicts with that hypothesis. Formation of a rapidly rotating magnetised neutron star (that is, a pulsar) during the collapse causes powerful optical emission from supernovae of this type. It is the pulsar that is pumping energy into the expanding and adiabatically cooling shell of the exploded star. Recent calculations⁴ have shown that such a 'slow' pumping of energy (as compared with the instantaneous explosion assumed in the previous models) can ensure the light curve observed for type I supernovae.

Since no sufficiently powerful optical emission was observed in the 'compact'-supernova event of 1668, we must conclude that, for some reason, it was not accompanied by the formation of a neutron star. It follows from X-ray radiation analysis of Cas A (ref. 5) that much gas (3 – $6 M_\odot$) was ejected during the explosion.

All these observations indicate that the star which exploded and gave birth to Cas A was a very massive object. The analysis of X-ray observations, for example, implies that the mass of the hydrogen-rich shell which the star lost in the pre-explosion stage was at least $10 M_\odot$. Therefore, when it had been on the main sequence its mass was not less than $20 M_\odot$. This accounts for the unique character of the Cas A source: although it is already

about 300 yr old, there is no source brighter than Cas A among a dozen younger sources in the Galaxy. Why it is unique is obvious: very massive stars seldom form.

The example of Cas A shows that high mass stars first detach their hydrogen-rich shells and then—after about 10^5 yr—they explode without giving birth to a neutron star and without the associated intense optical outburst. (Note that if a neutron star had formed after the Cas A explosion a point source of soft X-ray radiation would be observed there; a neutron star could not cool down to less than 3×10^6 K in 300 yr^6 .)

Thus after the explosion the star must either have completely dispersed, or most of its mass must have collapsed into a black hole. It is not yet possible to choose between these alternatives; but it is likely that in Cas A the gravitational collapse ended in the formation of a black hole. First, it follows from the relatively small mass of the hydrogen-depleted part of the exploded star (the part observed as fast moving filaments) as compared with the earlier-detached hydrogen rich shell. The chemical composition of the material in the fast-moving filaments is the most important argument favouring the conclusion that a black hole formed during the Cas A supernova event. The absence of elements of the Fe-group is highly characteristic of the spectrum observed for the fast-moving filaments. However, theoretical calculations of the star's expansion⁷ lead us to expect that at least $\sim 1 M_\odot$ of the exploded star should have transformed into Fe-group elements. In that case the spectrum of fast-moving filaments would inevitably have Fe II lines, in particular, the intense $\lambda 4570$ line.

Thus the observations strongly support the conclusion that a black hole formed after the Cas A outburst.

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Millimetre and submillimetre measurements of the Crab Nebula

THE flux from the central region of the Crab Nebula in three broad spectral passbands with flux-weighted mean wavelengths of $300 \mu\text{m}$, $400 \mu\text{m}$ and 1 mm have been measured. We report here that the inferred total flux densities for the entire nebula (Table 1) are consistent with an extrapolation of the power law spectrum found at longer wavelengths¹.

The 300 and $400 \mu\text{m}$ observations of the Crab Nebula were made with the University of Chicago $f/13$ photometer on the NASA Kuiper Airborne Observatory in September 1978. The detector was a Ga–Ge bolometer with heat trap field optics². The system had a focal plane aperture of $1.9'$ and a chopper throw of $5.0'$. The photometric pass bands were defined at short wavelengths by interference filters and at long wavelengths by diffraction and by the transmission characteristics of the light collector. We have measured the relative response of the photometer in the laboratory using both a grating spectrometer and a Fourier transform spectrometer. The two pass bands are shown in Fig. 1. W51 was used as the calibration source, assuming a spectrum of the form $\nu B_\nu(T)$ where $\pi B_\nu(T)$ is the

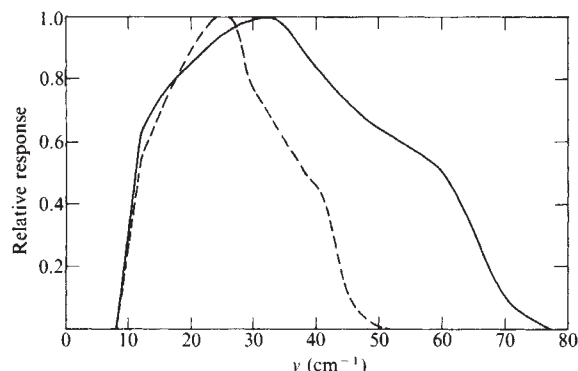


Fig. 1 Relative responses of the passbands used for airborne observations. The solid and dashed curves have mean wavelengths of 300 μm and 400 μm , respectively.

Planck function. For a 1.9' aperture centred on the submillimetre peak of W51, we have assumed $T = 40$ K and have normalised the function such that the flux density at 200 μm is 24,000 Jy. These parameters give a good fit to measurements in the range $80 \leq \lambda \leq 540$ μm (refs 3–5) allowing for the effects of beam size. The signal-to-noise ratios for the Crab Nebula observations were 9 for the 300 μm pass band and 3.6 for the 400 μm pass band.

The 1-mm measurement was made with the Chicago $f/7.5$ photometer at University of Hawaii 2.2 m telescope at Mauna Kea in September 1977. This detector also uses a Ga-Ge bolometer with heat trap field optics. The focal plane aperture and chopper throw were 3.2' and 8.0' respectively. The spectral response was defined by a helium temperature filter of fluoro-gold and black polyethylene, by the transmission of the atmosphere, and by diffraction. Jupiter was used as the calibration object with an assumed 750 μm brightness temperature of 162 K (ref. 6). The Crab Nebula observations had a signal-to-noise ratio of >6 .

We derived flux densities from the observed signals by the methods described in ref. 7. The spectrum of the Crab Nebula was assumed to be proportional to $\nu^{-0.26}$ in agreement with the radio spectrum; a change of ± 0.25 in the assumed spectral index produces a change of $<3\%$ in the derived flux densities. To allow for the effects of diffraction on the spectral response, we have assumed a gaussian source profile, convolved the assumed source with a diffraction pattern, and calculated the fraction of radiation within the focal plane aperture as a function of wavelength. The total flux densities in Table 1 were derived assuming a source size of $2.3 \times 3.6'$ FWHM[†]. Errors of $\pm 0.3'$ in both source dimensions result in errors of $\leq 10\%$ in the obser-

ved flux density, but lead to errors of 15–20% in the extrapolation to total flux density. The estimated error in absolute calibration is $\leq 20\%$.

Werner *et al.*⁹ have also measured the total flux density of the Crab Nebula at 1 mm. Their value, 300 ± 80 Jy, is much larger than ours. The discrepancy is due primarily to the different methods used to obtain the total flux density. Werner *et al.* used a 1' beam to map the source and derived a total flux density from integration of their map. Their map shows a source with FWHM of $4.5' \times 2.5'$, although this size is somewhat uncertain due to the low signal-to-noise ratio in the outer regions of the map. The larger size leads to a higher total flux density. If we derive instead a total flux density from their observed central surface brightness and our assumed source size, we get a value of 210 Jy, in much better agreement with the value in Table 1. The question of the source size at wavelengths ≤ 1 mm deserves further study.

The total flux densities in Table 1 are consistent with a continuation of the radio spectrum into the submillimetre region with no change in slope (Fig. 2) although the errors would allow a change in slope of 0.25. However, the present data can be used to exclude more radical departures from the extrapolated radio spectrum. Kirshner¹⁰, for example, has suggested that the radio spectrum might steepen above 15 GHz. The resulting submillimetre flux densities would lie almost a factor of 3 below our measured values.

The present data show no increase in flux density at short-wavelengths which could be attributed to thermal radiation from

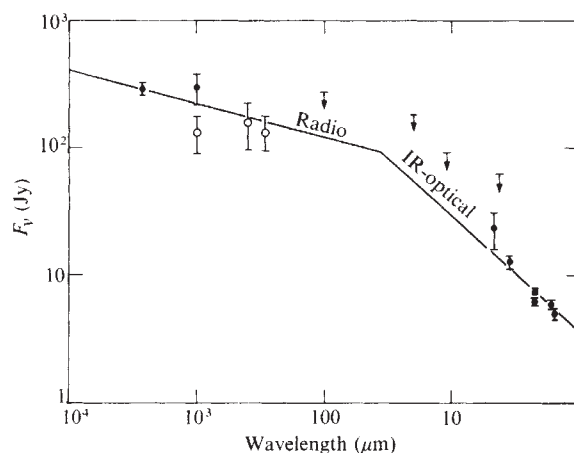


Fig. 2 Spectrum of the Crab Nebula. Data sources: $\circ$, this paper; 2.7 mm, ref. 8; 1 mm, ref. 9; 20 μm , 11 μm and 4.2 μm , ref. 12; 4.75 μm , 2.2 μm , 1.65 μm and 1.55 μm , ref. 13; 3.5 μm and 2.2 μm , ref. 14; 100 μm , ref. 15; radio and IR-optical fits, ref. 1.

Table 1 Submillimetre flux density of the Crab Nebula

Mean wavelength (μm)	Observed flux density (Jy)	Total flux density (Jy)
300	$35 \pm 8^*$	$135 \pm 41^\dagger$
400	$41 \pm 14^*$	$158 \pm 63^\dagger$
1,000	$75 \pm 19^\ddagger$	$131 \pm 42^\dagger$

* Into a 1.9' aperture. The error includes a 20% contribution from uncertainties in absolute calibration and in the assumed source spectrum.

† Assuming a gaussian source with FWHM of $2.3' \times 3.6'$. The error includes an additional 20% contribution due to the extrapolation to total flux density.

‡ Into a 3.2' aperture. The error includes a 20% contribution from uncertainties in absolute calibration, assumed source spectrum and atmospheric water vapour.

dust. From our measurement at 300 μm we may place an upper limit on the dust emission within the range $170 < \lambda < 900$ μm equal to the observed flux, $\nu F_\nu < 1.3 \times 10^{-12}$ W m^{-2} . For dust spectra which have their peaks in this wavelength range, corresponding to $T \leq 20$ K, our result improves the UCL limit of 2×10^{-10} W m^{-2} between 40 and 350 μm ¹¹.

At 100 μm the best limit on the flux of the Crab Nebula is $F_\nu < 275$ Jy¹⁵, which gives $\nu F_\nu < 10^{-11}$ W m^{-2} . We conclude that the luminosity of dust emission from the Crab Nebula is less than 1300 $L_\odot$ for dust with temperatures ≤ 40 K, but that there could be as much as $5 \times 10^3 L_\odot$ emitted by dust with $T \sim 120$ K.

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Acoustic absorption by MgCO_3^0 ion-pair relaxation

UNDERWATER sound propagation measurements in natural bodies of water frequently reveal attenuation anomalies that cannot be ascribed to known processes. When chemical relaxations are suspected, the acoustic resonator-decay method has proved an effective means of investigation in laboratory conditions. By stepwise synthesis of an artificial medium of similar composition and measuring changes in decay rates, the responsible chemical constituents can be identified. Then by combined stoichiometry and acoustic measurements with different concentrations, the relaxation kinetics of the reacting species can be determined. We have shown previously by resonator measurements that the acoustic absorption in Lake Tanganyika is the result of a chemical relaxation involving magnesium and carbonic acid¹. The same relaxation is also believed to be responsible for one of the absorption components in seawater². We show here that the relaxation kinetics follow a two-step association of the magnesium carbonate ion-pair.

If activity effects are neglected, the absorption arising from the slower second step of the two-step equilibrium $\text{A} + \text{B} \xrightleftharpoons[2]{1} \text{C} \rightleftharpoons \text{D}$ can be written

$$\alpha = \alpha_{\max} f^2 / (f^2 + f_r^2) \quad (1)$$

where f is the acoustic frequency and

$$\alpha_{\max} = \frac{(\Delta V)^2 k_2 K_2 (C + D)}{2\beta_0 RT(1 + K_2)} \text{ neper s}^{-1} \quad (2)$$

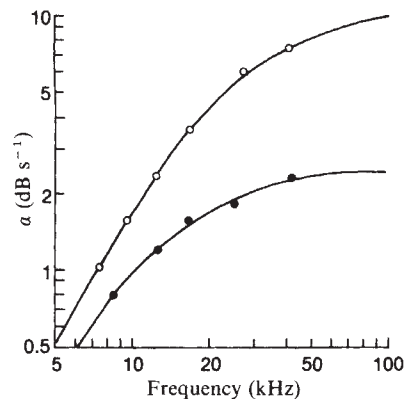


Fig. 1 Measured decay rates against frequency for $[\text{NaCl}] = 0.4 \text{ M}$ and $[\text{CO}_2] = 2.5 \text{ mM}$. ●, $[\text{Mg}] = 5 \text{ mM}$, $\text{pH} = 9.0$, $pK'_{2C} = 9.56$. ○, $[\text{Mg}] = 50 \text{ mM}$, $\text{pH} = 8.7$, $pK'_{2C} = 9.17$.

The respective concentrations are given by A , B , C and D and the equilibrium constants by $K_1 = C/AB$ and $K_2 = D/C$. The relaxation frequency is given by

$$f_r = \frac{k_2}{2\pi} \left[\frac{1 + K_1(1 + K_2)(A + B)}{1 + K_1(A + B)} \right] \text{ Hz} \quad (3)$$

where k_2^- is the reverse rate constant of step two and $K_2 = k_2^+/k_2^-$ where k_2^+ is the forward rate constant. The effective change in molar volume is given by $\Delta V = \Delta V_2 + \Delta V_1/(1 + K_1(A + B))$ where ΔV_1 and ΔV_2 are the molar volume changes of the respective steps. Other constants are: compressibility, $\beta_0 = 1/\rho_0 c_0^2$ where ρ_0 is density and c_0 is the speed of sound; gas constant, R and absolute temperature, T .

In the present case let $A = [\text{Mg}^{2+}]$, $B = [\text{CO}_3^{2-}]$ and $C + D = [\text{MgCO}_3^0]$. As $\text{pH} > 7$ is the domain of interest, the carbonate-bicarbonate equilibrium is the only concern⁴. The equilibrium constant is given by $K_{2C} = a_{\text{H}}[\text{CO}_3^{2-}]/[\text{HCO}_3^-]$ where a_{H} is the hydrogen ion activity.

To avoid inherent problems at low ionic strengths, all measurements were made in 0.4 M NaCl solutions. If other minor ion-pairings are neglected, the apparent equilibrium constant can be written⁵

$$K'_{2C} \approx K_{2C}(1 + K^*[\text{Mg}^{2+}] + K^*[\text{Na}^+]) \quad (4)$$

where $K^* = [\text{MgCO}_3^0]/([\text{Mg}^{2+}][\text{CO}_3^{2-}])$ and where $K'_2 = [\text{NaCO}_3]/[\text{Na}^+][\text{CO}_3^{2-}]$. From this we have

$$[\text{MgCO}_3^0] \approx [\text{CO}_2] \frac{x}{1+x} \frac{y}{1+y_0+y} \quad (5)$$

where $x = K'_{2C}/a_{\text{H}}$, $y = K^*[\text{Mg}^{2+}]$ and $[\text{CO}_2]$ is the total concentration. The apparent equilibrium constants were measured in a solution containing equal concentrations (1 mM) of NaHCO_3 and Na_2CO_3 which gives $K'_{2C} \approx a_{\text{H}}$ or $pK'_{2C} \approx \text{pH}$ (pH was measured by means of an Ag/AgCl electrode). The value $y_0 = K^*[\text{Na}^+]$ was determined from the change in K'_{2C} when the NaCl was added. The value of K^* was then determined from the slope of K'_{2C} against $[\text{Mg}^{2+}]$ measured when MgCl_2 was added in steps. In the range of concentrations being considered, we have approximated $[\text{Mg}^{2+}] \approx [\text{Mg}]$ where $[\text{Mg}]$ is the total concentration⁶.

Our attention has been drawn to ref. 6 which shows that MgCl^+ ion-pairing may also be important. To account for the reduced free ion concentration we can write $[\text{Mg}^{2+}] \approx \text{constant} \times [\text{Mg}]$. However, as K^* was measured in identical conditions, its value must be correspondingly increased which leaves the results of equation (5) unchanged.

All measurements were made at 22 °C. The resonator apparatus and procedures are described in ref. 2.

Figure 1 shows typical measurements of decay rates against frequency. The data are fitted by equation (1) which gives $\alpha_{\max}$ and f_r for each set of experimental conditions. The $\alpha_{\max}$ values were normalised by the formula $\alpha_{\infty} = \alpha_{\max}(1+x)/x$. Thus α_{∞} is the value one would have for $pH \gg pK_{2C}$, that is, if all CO_2 were in the form of the three carbonate species. The values were converted from $dB\ s^{-1}$ to $Np\ s^{-1}$ by dividing by 8.7.

The α_{∞} and f_r data are shown in Fig. 2. The f_r data were fitted by equation (3) using the measured value $K_1^* = K_1(1+K_2) = 160/M$ with $K_2 = 2$ and $k_2 = 6 \times 10^4\ s^{-1}$. The α_{∞} data were fitted

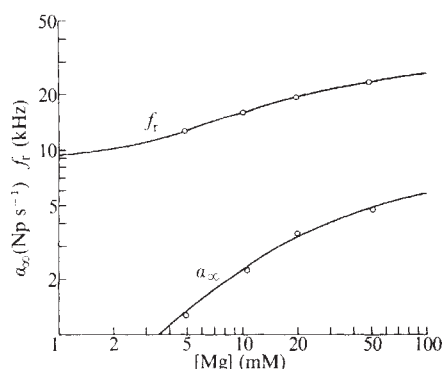


Fig. 2 Absorption α_{∞} and relaxation frequency f_r plotted against magnesium concentration for 0.4 M NaCl solution. Similar curves would be obtained for Lake Tanganyika conditions except that the $[Mg]$ scale would be roughly an order-of-magnitude smaller because of the much greater values of K_1^* at low ionic strengths.

using the value $\Delta V = \Delta V_2 = 12\ ml/M$ in equation (2) and the measured value $y_0 = 3.5$ with $x \gg 1$ in equation (5).

The agreement between theory and experiment shows that the absorption mechanism in question can be modeled as the two-step $MgCO_3$ equilibrium in which the slower second step controls the relaxation process. The calculated rate constants should be valid for both Lake Tanganyika and seawater at 22 °C. It appears, therefore, that the earlier estimate of the Lake Tanganyika relaxation frequency (40 kHz) is too high. Present measurements indicate that it should be no greater than 30 kHz.

Calcium ion-pairing may not entirely account for the reduced absorption when $CaCl_2$ is added^{1,2}; hence, a decrease in ΔV_2 may also be involved. In seawater, calcium evidently provides the coupling between the magnesium carbonate and the boric acid/borate equilibria that is responsible for an order of magnitude increase in the relaxational absorption of the latter⁷.

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Anomalous characteristics of the microcrystalline state of SiC fibres

FIBRES of SiC are prepared¹⁻⁴ from the polycarbosilane which, in various forms, is made into a precursor by melt-spinning: the precursor is then heated to produce the SiC fibre. Ceramics are produced in this way by heating the organosilicon polymer. The organic precursor is prepared in a subtle form (for example, as fibres) which is extremely difficult to produce from an inorganic precursor. On heating the organic precursor a ceramic skeleton remains. This inorganic residue derived from an organosilicon polymer possesses characteristics not previously known and these are reported here.

Table 1 shows a chemical analysis of the SiC fibre obtained after heat-treatment (1,300 °C) of the precursor fibre. Its atomic ratio is Si:C:O:H = 1:1.46:0.36:0.03. Table 1 shows that the SiC fibre contains appreciable amounts of carbon and of oxygen which had been introduced by the heating of the precursor fibre. Assuming then that the oxygen is present in the fibre as SiO_2 , the molar ratio of compounds in the SiC fibre is $SiC:C:SiO_2 = 1:0.78:0.22$.

The apparent density of the β -SiC fibre obtained by heating a precursor fibre at 1,300 °C is $2.6\ g\ cm^{-3}$, which is lower than the true density $3.2\ g\ cm^{-3}$ of β -SiC.

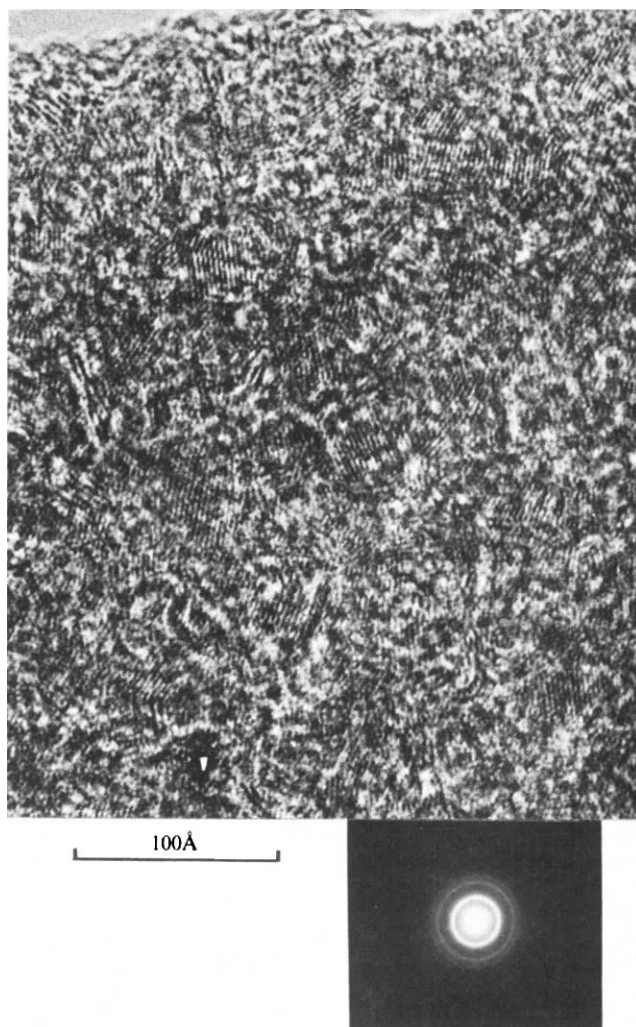


Fig. 1 High resolution electron micrograph of a precursor fibre heat-treated at 1,300 °C.

Table 1 Composition (wt%) of fibres

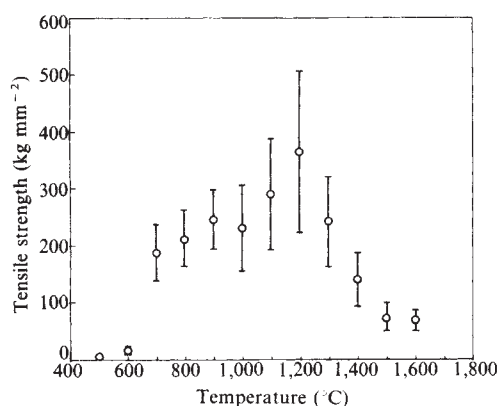
	Si	C	O	H
Precursor fibre	46.8	38.1	0.82	7.40
Fibre after curing	44.4	31.0	15.5	5.30
SiC fibre	50.5	31.6	10.3	0.055
SiC fibre after heat treatment at 1,250 °C	51.3	23.9	18.5	0.015

The SiC fibre possesses a high tensile strength (350 kg mm^{-2}), a diameter of $10 \mu\text{m}$ and a high Young's modulus ($20 \text{ tonnes mm}^{-2}$). The fibre sustains a load of 200 kg mm^{-2} at $1,250^\circ\text{C}$ for 3 d in air. Table 1 shows a chemical analysis of the SiC fibre after heat-treatment. Its compound molar ratio is $\text{SiC}:\text{C}:\text{SiO}_2 = 1:0.59:0.46$; a lot of carbon still remains.

The tensile strength and the Young's modulus of the SiC fibre are independent of temperature (between room temperature and $1,400^\circ\text{C}$) *in vacuo*⁵, and the strength at the high temperature in air⁶ is as good as other silicon carbide fibres^{7,8}.

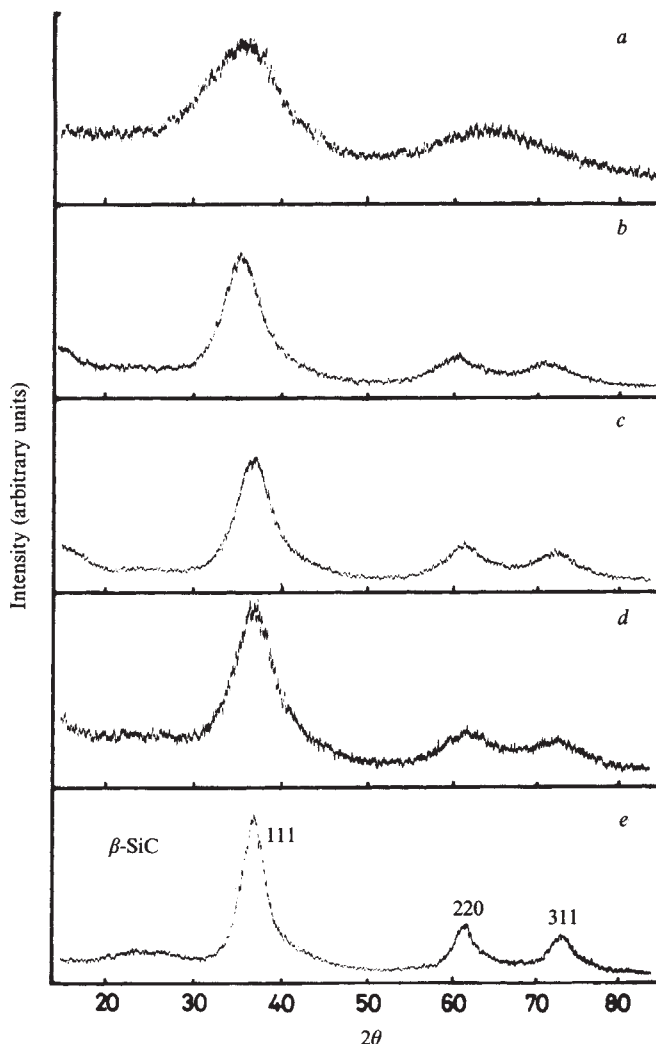
SiO_2 is considered mainly to exist at the surfaces of the SiC fibre. We investigated how the SiC and the excess carbon come to exist in the fibre.

It has been previously reported that in the SiC fibre, the SiC matrix is distributed uniformly as fine grains from the result of X-ray diffraction¹⁻⁴. Figure 1 shows a high-resolution electron

**Fig. 2** Variation of the tensile strength of precursor fibres with heat-treatment temperatures.

micrograph of the powdered SiC fibre. The fibre was prepared by heating a precursor fibre at $1,300^\circ\text{C}$ in a vacuum. The lattice image corresponding to a $2.5 \text{ \AA}$ interlayer spacing of $\beta\text{-SiC}$ (111) planes and a $3.4 \text{ \AA}$ interlayer spacing of graphite (002) planes are observed. The SiC is ultrafinely grained. Figure 1 also indicates a uniform distribution of fine grains of carbon surrounded by $\beta\text{-SiC}$. It is difficult for the carbon in this condition to be oxidised. A high-resolution electron micrograph of SiC fibre after the $1,250^\circ\text{C}$ heat-treatment was also taken. The distribution of carbon was almost the same as before the heat treatment.

The excess carbon and the oxygen introduced in the curing process may affect the microcrystalline state of $\beta\text{-SiC}$. The relationship between the mechanical strength of the fibre and the heating temperatures for the precursor fibre *in vacuo* is shown in Fig. 2. Heating at $1,500^\circ\text{C}$ causes the mechanical strength to drop, as shown in Fig. 2. Figure 3 shows the X-ray (Ni-filtered $\text{Cu K}\alpha$) diffraction patterns of the precursor fibre heated at $1,000$, $1,100$, $1,200$, $1,300$ and $1,500^\circ\text{C}$. The X-ray diffraction patterns on heating at $1,500^\circ\text{C}$ indicate a larger fraction of crystallised $\beta\text{-SiC}$ in comparison with the patterns of fibres heated at $1,100$ – $1,300^\circ\text{C}$. The mechanical strength drops with crystallisation of the $\beta\text{-SiC}$. The stable and uniformly distributed carbon in the SiC fibre may be precipitated with

**Fig. 3** X-ray diffraction patterns of precursor fibres heat-treated at various temperatures. Full-scale intensity of patterns at $1,100$ (b); $1,200$ (c); and $1,500^\circ\text{C}$ (e) is twice that of patterns at $1,000$ (a) and $1,300^\circ\text{C}$ (d).

crystallisation of the $\beta\text{-SiC}$ phase, hence lowering the mechanical strength: in other words, the SiC fibre in the microcrystalline state has high mechanical strength.

Chemical analysis of the SiC fibre and its high-resolution electron micrograph show an unexpectedly large quantity of excess carbon existing in the fibre. In spite of this, this fibre has a high strength and a high heat-resistance at high temperatures.

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Spontaneous formation of lecithin bilayers at the air-water surface

THE energetics of formation of various surface film states of dimyristoyl lecithin (DML) has been recently obtained from equilibrium spreading pressure π_e measurements¹. One result from that study, but not reported then, was the formation of a new equilibrium lecithin surface phase. Its existence was deduced solely from formal thermodynamic arguments based on the Gibbs phase rule, and therefore reporting of the new surface phase was deferred until more direct experimental evidence was available for describing its properties. We have now characterised this new surface phase by measuring the surface concentration of DML films and have found the concentration to be double that of a close-packed monomolecular film. Before presenting these results and their implications, we summarise the arguments which led to the prediction of the new lecithin surface phase.

In principle, π_e measurements as a function of temperature indicate the existence of all phase transitions; they also provide a thermodynamic basis for calculating transition energies²⁻⁴. The equilibrium surface phase relations for DML obtained by this approach in the region of T_c , the gel-liquid crystal transition temperature, is given in Fig. 1a. Line ABCD represents the coexistence line for the equilibrium of surface film with bulk DML, either gel or liquid crystal. Point B is at 23.5 °C, T_c for DML. At temperatures below T_c , the bulk gel state is in equilibrium with a gaseous surface film; at temperatures above T_c liquid crystal is in equilibrium with a gaseous film which changes with increasing temperatures into a liquid expanded film along line CD. Line CE represents the equilibrium of liquid expanded film with surface gas in the absence of bulk lipid; these values of π are the equivalent of the surface vapour pressure¹. Line CE' represents the supercooled liquid expanded film surface vapour pressure; it is formed when solvents are used to form the film at temperatures below 25 °C (ref. 1).

The phase line CD which describes the equilibrium between liquid crystal and liquid expanded film indicates that π_e increases rapidly with temperature. But π_e must reach a finite limit at some elevated temperature. Indeed, measurements of π_e for DML at temperatures exceeding 25 °C, shown in Fig. 1, indicate that π_e reaches a maximum of 49.5 dyn cm⁻¹ at about 29 °C. With further increase in temperature π_e decreases slightly. Similar results were obtained by Phillips and Hauser⁵.

The Gibbs phase rule at constant atmospheric pressure for this system⁶ may be written

$$F = C - P_s - P_b + 2$$

where C is the number of components (water, lecithin, and air), P_s and P_b are the number of surface phases at the air-water surface, and bulk phases, respectively, and F is the number of intensive variables (temperature and chemical potential) which may be varied independently. For this system, with bulk lipid and at least one surface phase always present, F is either 1 or 0. Thus, when π_e varies with temperature, $F = 1$ and the chemical activity of lipid in each equilibrium phase also varies with temperature. Examples are given in Fig. 1a, along line AB where $P_b = 3$ (gel, water, air), $P_s = 1$ (surface gas), and along line BC where the bulk lipid gel state becomes liquid crystal at the elevated temperatures. At the gel-liquid crystal transition temperature, point B (Fig. 1), $F = 0$, as $P_b = 4$ (gel, liquid crystal, water, air), and $P_s = 1$.

At temperatures above and below 29 °C (the temperature where π_e is a maximum) π_e is also temperature dependent, $F = 1$, and therefore homogeneous surface films exist both below and above 29 °C. However, at 29 °C $F = 0$, and the phase rule therefore indicates that a phase transition occurs just as at

point B (Fig. 1); this new phase transition may be either in the surface or bulk. Because the temperature where this occurs exceeds T_c , the only lipid bulk phase present is the liquid crystal. As there is no evidence for a DML bulk phase transition at 29 °C, we deduce that at the temperature where π_e is a maximum, DML forms a new surface phase. To establish that the new phase which appears at 29 °C is in the surface, DML surface concentrations were measured by two independent methods.

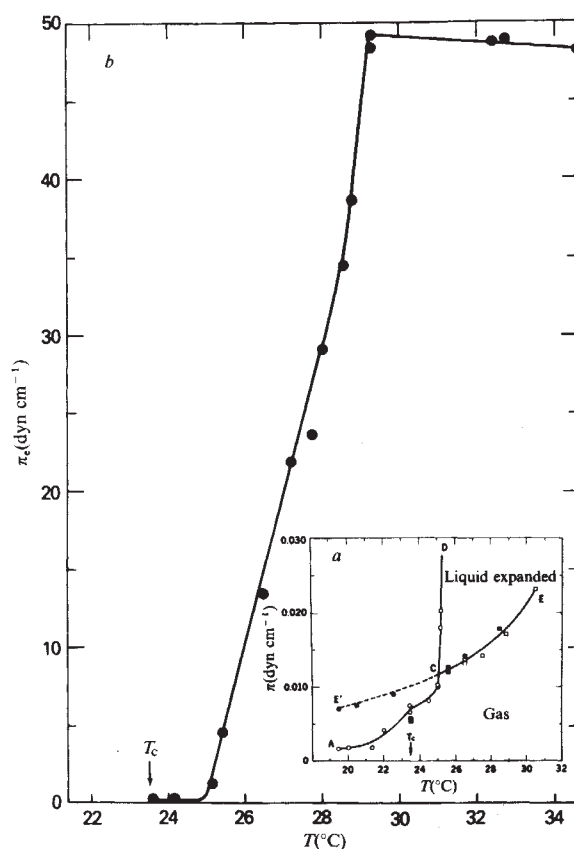


Fig. 1 a, Surface pressure π as a function of temperature in the region of the gel-liquid crystal transition temperature, T_c for DML. For explanation of phase diagram see text, and ref. 1. b, Complete equilibrium spreading pressure π_e -temperature diagram for DML. Note change in scale for π . Wilhelmy plate method used for this data, sensitivity of the order of 0.1 dyn cm⁻¹. Data for the figure inset were obtained by the millidyne balance¹. Therefore the details seen in the inset are not observed in the expanded range figure.

The first method entails isolating and collecting the surface film which accumulates in the air-water surface when DML crystals are placed on the water surface. A temperature controlled ($\pm 1^\circ$) film balance trough was divided into two compartments by a strip of Teflon. The strip has a slit on its upper edge to allow a small connection at the surface between the two water compartments. Bulk lipid was placed on the water surface of one compartment and held in place by a platinum wire loop; the second compartment was monitored for changes in π by the Wilhelmy plate technique. The bulk lipid generally remained effectively trapped behind the Teflon strip separating the two compartments. Films which accumulate at the surface of the second compartment were swept by a Teflon barrier over the edge of the trough and across a collection spout into beakers. The water which is entrained with the lipid amounts to about

0.01 ml cm⁻² of surface film⁷. This water was evaporated and the dried phospholipid was analysed for phosphorus⁸. Generally 350–400 cm² of surface was sufficient to provide enough lipid for the analysis. The films were collected 2 and 4 h after the surface pressure had ceased to change; the time interval had no significant influence on the amount of material collected.

For the second method the surface concentration was obtained by measuring the surface radioactivity of ³H-DML adsorbed from equilibrium dispersions of DML. The dispersions of DML gave identical values of π_e as those obtained by spreading from the crystal. A thermostatted ($\pm 0.1^\circ$) glass cell was used for both the π_e and radiotracer measurements of the dispersions. Specific activity of ³H-DML was approximately 35 Ci mol⁻¹, (a purified sample provided by Dr R. E. Pagano). Surface radioactivity was monitored by an end-window gas-flow detector; the window was composed of Parylene⁹ with a vacuum deposit of gold (approximate thickness: 70 μ g cm⁻¹ Parylene, 30 μ g cm⁻¹ gold). The detector was constructed by the Aloka Company, Mitaka, Japan, and was used with a standard scalar (Nuclear-Chicago, model 168). The counting efficiency of the detector was about 2% at a height of 1 mm above the water surface. Surface radioactivities were not affected by bulk particles of DML in the dispersion because the lipid density is greater than for water¹⁰, thus these particles settled out of the range of tritium ($\sim 1 \mu$ m in water) within 30 min. More complete details of the method will be presented elsewhere. Dispersion concentrations of 0.1–1.0 mg ml⁻¹ were used and the reported surface radioactivities were found to be independent of the dispersion concentrations.

The surface concentrations of DML as a function of temperature obtained by both methods is shown in Fig. 2. The values of Γ obtained by the two methods are in general agreement, with the larger error of the sweeping experiments reflecting the multiple operations required for the analysis. Below 25 °C where the equilibrium surface pressure is very low (surface gas region, Fig. 1) the surface concentration is close to zero. With increasing

temperature, the surface concentration increases to a maximum value of 6.4×10^{-10} mol cm⁻² at about 29 °C. At still higher temperatures Γ decreases monotonically to 3.2×10^{-10} mol cm⁻² at about 35 °C.

Film balance studies indicate that close-packed monolayers occupy 50–55 Å² per molecule or $3.3\text{--}3.0 \times 10^{-10}$ mol cm⁻² (ref. 11). Thus the adsorbed film formed at 35 °C (Fig. 2) appears to be a close-packed monolayer; at 29 °C the surface concentration is exactly double that of the close-packed monolayer, or the equivalent of a bilayer. The formation of the surface bilayer is not fortuitously dependent on the experimental conditions because its formation is independent of the dispersion concentration, and the same results were obtained by two independent methods. Moreover, the temperature of surface bilayer formation coincides with the temperature in which π_e is a maximum (Fig. 1b), and for which the phase rule predicts that a new lecithin phase appears. The new surface phase predicted by the phase rule is the surface bilayer.

While we cannot ascribe molecular configurations to the surface films which are reported in these studies, hypotheses consistent with the phase rule are suggested for describing the surface phase transitions. Figures 1 and 2 indicate that DML surface films between 25 and 29 °C pass continuously from the gaseous to the bimolecular state. As the phase rule predicts that a single homogeneous surface phase is present, we assume that at each temperature in this interval states of aggregation intermediate between gaseous and bilayer are formed. Between 29 and 35 °C the data suggests that the surface bilayer reverts to a condensed monolayer structure. In this interval the phase rule indicates that the surface film is homogeneous, but that surface bilayer must also be present. We therefore deduce that bilayer is transformed continuously to condensed monolayer, but assume that monolayer which forms is completely miscible with the bilayer, that is both monolayer and bilayer coexist in a homogeneous surface phase. At temperatures exceeding 35 °C condensed monolayer is the only surface state present. Phenomenologically, DML forms in sequence with increasing temperatures: gaseous film, surface bilayer and finally condensed monolayer.

Several additional aspects of this system are noteworthy. The surface bilayer will form only in the presence of bulk phase liquid crystal, and at a unique temperature which is presumably a characteristic of each lecithin. In equilibrium conditions the surface bilayer will not exist independently of multilamellar liquid crystal. Questions on the generality of these phenomena and the thermodynamics of formation of surface bilayers will be examined in detail elsewhere. Finally, it has always been a basic tenet of lipid film studies on water that the films may be assumed to be monomolecular. We have shown that for at least one equilibrium system, surface films can exceed monolayer concentrations.

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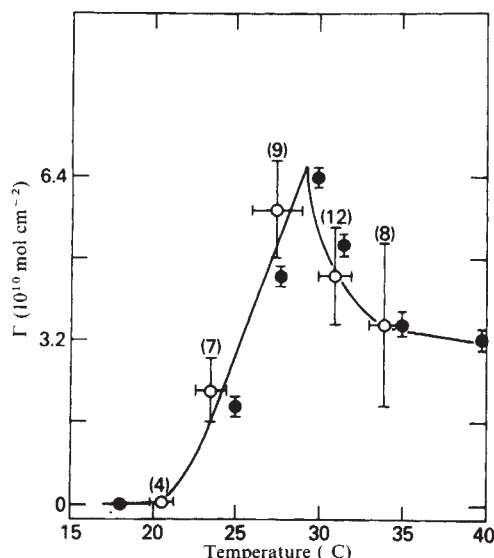


Fig. 2 Surface concentration of DML films as a function of temperature. ○, Film concentration obtained by sweeping method, films formed by spreading from bulk crystals. Each data point is given with standard errors of the mean, and the number of samples are shown in parentheses above the error bar. The large s.e.m. reflect the multiple operations required for the analysis and the poor temperature control ($\pm 1^\circ$) inherent in the large film balance trough used. ●, Film concentration obtained by radiotracers, films formed by adsorption from dispersions containing ³H-DML. Duplicate analyses gave a reproducibility of $\pm 5\%$, temperature controlled to $\pm 0.1^\circ$.

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Thermoluminescence dating of a deep-sea sediment core

IN the last decade thermoluminescence dating has been developed for use on archaeological material, principally pottery, that was heated in antiquity¹. Thermoluminescence (TL) is the light emitted by a material when heated and which results from a previous dose of radiation. In the simplest cases the light intensity is proportional to the radiation dose and can be used for determining an unknown dose; when combined with other measurements which yield the dose rate the TL can thus be used to calculate an age. In the case of pottery the event being dated is the last heating of the material to a high temperature, typically 500 °C. The use of TL to date the deposition of ocean sediments which we propose here is similar in the main principle except for the lack of the heating event. We describe here the experimental evidence which indicates that some event which has the same result does occur. We then show that exposure to sunlight could be this event, and finally show that TL dates obtained for an ocean sediment core are in agreement with dates determined independently.

The results presented here are for 4–11- μ m grains of samples of the deep-sea core RC8–39 from the Crozet Plateau of the Antarctic Ocean (long 42°21'E, lat 42°53'S, water depth 4,330 m). The sample preparation and measurement techniques are based on those of Zimmerman² and will be described in detail elsewhere.

Figure 1a shows a typical glow curve obtained for the sample at 580 cm below the top of the core. All the other samples gave similarly shaped glow curves but with an overall increase of TL intensity with depth down the core. A convenient measure of the TL intensity is the γ -radiation dose, D , that produces the same TL; we determined this using the additive dose method¹ in which the TL of the samples is compared with the TL of identical samples which have also received a laboratory radiation dose. Figure 2a shows the value of D obtained for each sample at the 300 °C glow-curve temperature, and that D increases with depth down the core.

A similar increase of TL with depth was previously reported in two North Pacific cores by Huntley and Johnson⁴. They thought that the TL was coming from the separated siliceous tests; however, we have since discovered that it was coming primarily

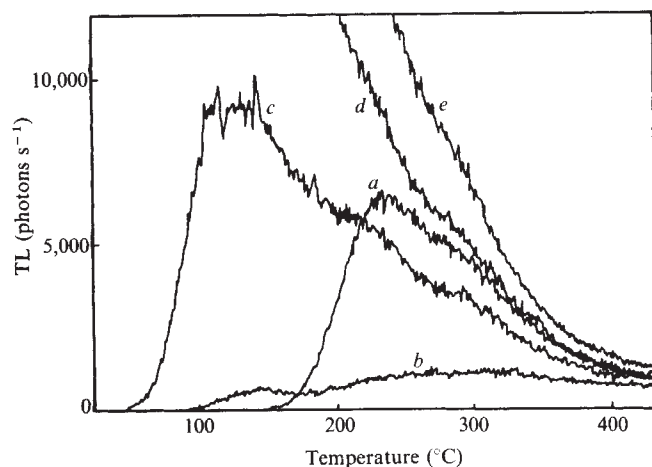


Fig. 1 Thermoluminescence glow curves from RC8–39 580 cm: *a* natural; *b* natural sample after a 40-min sunlamp exposure, *c–e* as *b* but after additional γ doses of 7.4, 14.8 and 22.2 krad respectively.

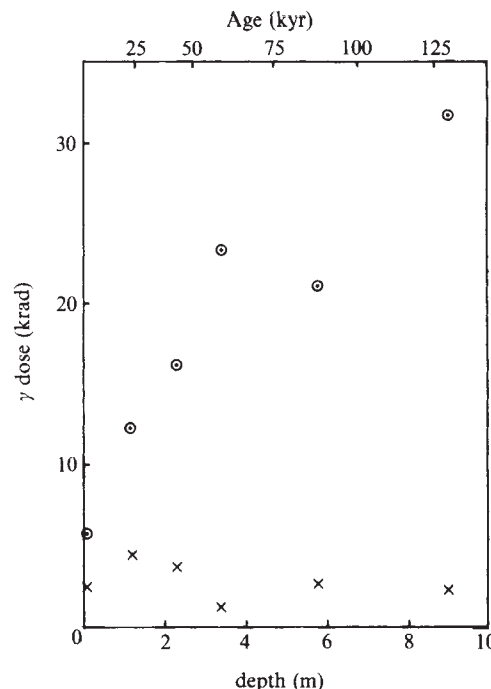


Fig. 2 ○, The TL intensity against depth for RC8–39 evaluated at a glow-curve temperature of 300 °C. D is the γ dose which produces the same TL as observed in the natural samples and was obtained by the additive dose procedure. ×, Values of D_0 for the same samples.

from the inorganic sediment still attached to the siliceous tests. In all three cores the TL of samples near the top of the core is significantly less than the TL of samples with an age of $\sim 10^5$ yr. We have found a similar low TL intensity in four other core top samples we have studied. We deduce, therefore, that the TL of freshly deposited sediment is small, and not in saturation as one might expect for material derived from terrestrial sources.

Further evidence for a low TL level in freshly deposited sediment is found in the TL studies of Quaternary sediments in the USSR^{5–9} and China¹⁰. Here an increase of TL with depth was also observed. In the original Soviet work Morozov⁵ and Shelkopyas⁶ suggested that the effects of sunlight and grinding during weathering were responsible for the low level of TL in freshly deposited sediments. Consequently we have examined the effects of sunlight on the TL of our samples and found a remarkable sensitivity: 20 min of exposure is enough to halve the natural TL.

To test the proposal that exposure to sunlight is a viable mechanism for setting the natural thermoluminescence to near zero we gave a set of identical samples a large γ dose (~ 400 krad), to bring them near saturation, and then measured the TL as a function of light exposure. This experiment was performed with a Sylvania sunlamp which is about four times as efficient as sunlight so that reproducible exposures could be obtained. The results, in Fig. 3, show that an exposure equivalent to ~ 10 d of direct sunlight is capable of reducing the TL to the level found in a sample at the top of the core. Hence we suggest that previously acquired geological TL could be erased by exposure to sunlight before deposition on the ocean floor. However, we have not carried out any detailed mineral separation and chemical and mechanical alteration during weathering and sedimentation may play a significant role as well. Whatever the cause of the low TL in freshly deposited sediment the following arguments may still be applied.

As Fig. 3 shows, even after long light exposures there is a finite TL signal. (This may be due to the presence of a TL signal that cannot be bleached easily or may be due to a non-radiation-induced signal.) We therefore describe the natural TL of a sediment sample by

$$I_{\text{nat}} = I_o + I_d \quad (1)$$

where I_o is the TL at the time of deposition and I_d is the TL due to the radiation dose since deposition. Ideally a core top sample should yield a TL signal I_o . In practice, however, its TL may also be expected to have a small I_d component due to the real age of the material because of loss of the most recent sediment during coring and because of sediment mixing due to bioturbation¹¹.

Rewriting equation (1) in terms of the γ doses that produce the same TL intensities we have

$$D = D_o + ED \quad (2)$$

where ED , the equivalent dose, is that laboratory γ dose that produces the same TL intensity as did the radiation dose experienced by the sample since deposition.

To determine ED we have developed the procedure shown in Fig. 1. All measurements are made on separate identical samples. Curve a is the TL of a natural sample. Curve b is the TL of a similar sample exposed to the sunlamp for 40 min. Curves $c-e$ are the TL values of samples exposed to the sunlamp and then given various γ doses. Figure 1 shows that a γ dose of about 13 krad would give a TL similar to that of the natural sample above 250 °C. This dose is called G . Figure 3 shows that the 40-min sunlamp exposure was not sufficient to remove all of the I_d component of the natural TL. Let us call the fraction that remained f . Then we have

$$ED = \frac{G}{1-f} \quad (3)$$

We have determined f in separate experiments in which samples were first given long sunlamp exposures, γ irradiated, and then given 40-min sunlamp exposures. The values of f , typically 0.2, did not depend measurably on the γ dose or core sample.

We tested the above procedure for obtaining ED by using it to determine the dose for samples which had been given a known laboratory dose after a substantial sunlamp exposure. As an additional test we have taken several sets of the 119 cm samples, given them known γ doses, Γ , in addition to the natural dose, and then used the above procedure to determine G for each set. The plot of G against Γ was linear and when extrapolated to $G = 0$ yielded a value $\Gamma = -ED$ independent of temperature above 250 °C and in accord with the one obtained by the

previous method. The above tests gave agreement within $\pm 5\%$.

The equivalent doses we have determined using equation (3) for six samples of RC8-39 are listed in Table 1. The increase with depth closely parallels that shown in Fig. 2. Values of D_o were obtained, by subtraction ($D_o = D - ED$), and those at 300 °C are shown in Fig. 2; they show no systematic variation with depth and therefore are consistent with the model.

From the equivalent doses listed in Table 1 we have calculated TL ages using radiation dose rates determined by the standard techniques worked out for TL dating of archaeological material¹. The age equation we have used is

$$ED = (R_K + R_U + R_{Th})T + R_1\tau\{\exp(T/\tau) - 1\} \quad (4)$$

where T is the age, the R values are the effective radiation dose rates due to the decay of ^{40}K , ^{238}U , ^{232}Th and ^{230}Th respectively and include α , β and γ contributions. The last term, which is dominant arises from the presence of excess ^{230}Th which is continuously precipitated from the ocean; the ^{230}Th mean life τ is 108 kyr and thus it is necessary to include the variation of dose rate with time. Each term in the equation includes a water

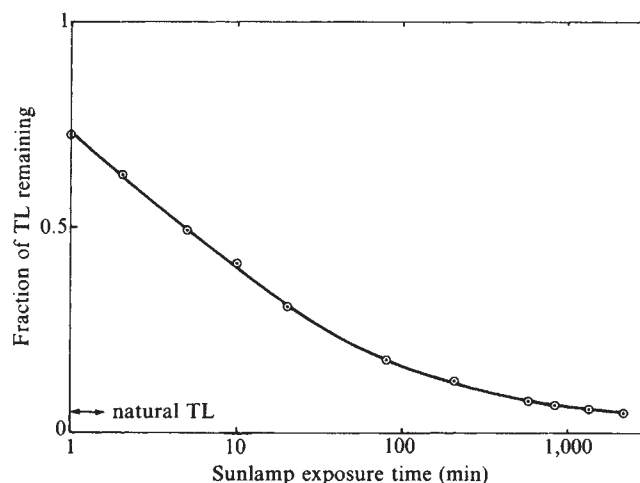


Fig. 3 The fraction of the TL evaluated at a glow-curve temperature of 300 °C remaining after a sunlamp exposure against exposure time. The experiment was performed on a set of samples which had been given a laboratory dose of 400 krad to simulate their condition before weathering.

attenuation factor calculated using an estimated water content of $57 \pm 5\%$, and each α dose-rate term includes a measured TL efficiency factor¹². The ^{238}U , ^{232}Th and ^{230}Th contents were determined using α -scintillation counting and the dose rate conversion factors calculated according to the method described by Bell¹³.

The ages thus calculated are shown in Table 1 where they are compared with those we have deduced from the *Cycladophora davisiana* and oxygen-isotope variation curves of Hays *et al.*³. We consider the correlation between the two sets of dates to be sufficiently encouraging to suggest that the method is basically sound. Complete details of the measurements will be published elsewhere.

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Table 1 Doses, radioactivity analyses, TL ages and *C. davisiana* ages for RC8-39

Depth (cm)	Equivalent dose (krad)	K content (%)	Measured α -count rate (ks cm ⁻²) ⁻¹	TL age (kyr)	Age from <i>C. davisiana</i> (kyr)
9	3.0	0.33	1.83 ± 0.03	9	6-10
118	7.2	0.82	1.25 ± 0.02	30	22-25
230	10.6	0.55	0.98 ± 0.02	51	40-46
342	19.1	0.71	1.02 ± 0.03	≥ 76	58-66
580	15.7	0.42	0.77 ± 0.01	85	85-90
902	25.6	0.53	0.56 ± 0.01	140	125-135

The *C. davisiana* ages were estimated by matching the *C. davisiana* variations shown in Figs 2, 3 and 5 of Hays *et al.*³. The known experimental uncertainty in the TL ages is about 18%, arising primarily from the stated uncertainty in the water content. The TL age for the 342 cm sample is shown as a lower limit because it showed anomalous fading; the others did not.

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Albedo contrast and glaciation due to continental drift

A POPULAR theory^{1,2} holds that glaciation occurs when landmasses drift over the poles and cool the Earth. However, the Earth cools on balance when landmasses bunch in the tropics, rather than over the polar caps. I suggest here that a peculiar distribution of land may thus be required during glacial periods, perhaps explaining their rarity³ and the long intervals when land was over the poles but the Earth was not glaciated⁴.

The conventional argument⁵ assumes that the Earth cools when landmasses of variable but high albedo displace water of low albedo at the poles. While snow, which has very high albedo, is more likely to accumulate on land than on water, the first snow must fall on a 'dark' landscape. A study of the atmospheric and intrinsic determinants of surface albedo, and of the best measurements, shows that the land–water albedo contrast is greatly attenuated at the poles, and indeed is greater in the tropics, for any plausible state of affairs not involving snow.

For unidirectional radiation, received directly from the Sun, the albedo of a smooth surface is inversely proportional to $\sin E$, where E is the angle of elevation of the Sun^{6,7}. Under perfectly diffuse radiation, the same surface has a different albedo, not dependent on E . The surface irradiance Q_* is never unidirectional; the atmosphere scatters the direct beam from the Sun and increases the diffuse fraction q_d of Q_* , the more so as E approaches 0° and the path length through the atmosphere increases. Solar radiation is never perfectly diffuse, but it is virtually so when the Sun is just below the horizon, and also under low overcasts.

As well as depending on E , the reflectivity of many surfaces varies with roughness. The choppier the sea, or the taller the plants⁸, the less the albedo, because multiple reflection between surface elements increases the chance of eventual absorption. Multiple reflection also reduces the albedo at high E , reinforcing the theoretical dependence mentioned above. These opposing tendencies, and that of q_d to increase at low E , make empirical facts essential.

For water the data of Grishchenko^{7,9}, for 0–25% cloud and waves of height <0.7 m, are outstanding. His albedos are much lower than those predicted for direct radiation incident on a

plane, rising from 4 to 8% at large E to a peak at $\sim 39^\circ$ around $E = 6-8^\circ$, then dropping to about 12–14 at $E = 0^\circ$. These low albedos can be taken to mean that Q_* becomes almost entirely diffuse at low E . For plant albedo, the following reliable ranges (in percentage points) are available:

- 3 (grass, $E = 70^\circ$ to 15°)¹⁰
- 8 (grass, $E = 55^\circ$ to 10°)^{7,11}
- 15 (apple tree, $E = 80^\circ$ to 10°)¹²
- 10 (Scots pine, $E = 65^\circ$ to 0°)¹³

Tooming¹¹ found a decrease similar to Grishchenko's below $E \approx 10^\circ$. It seems that plant albedo varies much less with solar elevation than does water albedo.

This implies that, when the Earth is free of snow, the land–water albedo contrast is largest in low latitudes. As E decreases polewards, high-latitude waters will have albedos considerably greater than low-latitude waters, while landmasses, presumably vegetated, will increase in albedo much less towards the poles. The best way to cool the Earth in times of benign climate is therefore to place as much land as possible in the tropics.

I have tested this proposition by planimetry of land areas in 10° latitude belts on palaeogeographic maps^{14,15} at 20-Myr intervals from about 240 Myr to 0 Myr. Sea level is assumed constant. These maps suffer from doubts as to the timing and geometry of drift; placement errors of the order of 10° (1 latitude interval) must be suspected, although their global sum will approach zero. Near the pole, albedo changes rapidly and fine resolution is desirable. I have calculated at 10° resolution, but have lumped the data into tropical and polar zones which are wide relative to the possible errors.

Figure 1a shows that the tropics have become much less watery in the past 80 Myr. The Earth has grown colder throughout the Cenozoic^{4,16}, and the cooling is thought to have accelerated, just as the calculated increase in tropical landmass has. The tropical landmass has recently reached and surpassed a

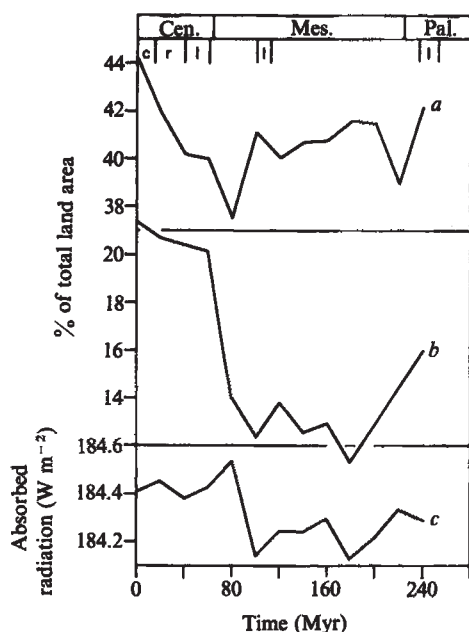


Fig. 1 a, Landmass between 30° N and 30° S; b, landmass polewards of 60° N and 60° S (both as % of total land area $\approx 148 \times 10^6$ km²); c, world average absorbed solar radiation (equation (1)). At top of graph c, l, r, stand for continental, regional and local glaciation respectively^{3,16,18}. The age of 240 Myr data points is merely convenient; the map is dated at 250 ± 25 Myr (ref. 15), and may or may not represent the early Permian glacial period. Errors of reconstruction are discussed in ref. 14; mean palaeomagnetic poles have 95% confidence limits of from 8.7° to 4.5° ; error in planimetry (sum of 10° -belt measurements/ 148.0×10^6 km²) on the several maps was from $+1.6\%$ to -1.6% .

size last attained in the Palaeozoic. Figure 1b, for comparison, shows an increase during the Cenozoic in the land surface polewards of 60° N and 60° S. Antarctica has moved little for 80 Myr¹⁵ or even 100 Myr¹⁷, but it has been glaciated only since 40 Myr, with an ice-sheet since 12–15 Myr⁴. North-east Siberia was over or near the North Pole throughout the Triassic and Jurassic, periods for which no one has found any glacial evidence anywhere. Ice-rafted detritus is, however, found in north-east Siberia in the late Permian¹⁸. Gondwanaland was over the South Pole until ~30 Myr after the Permo–Carboniferous glacial period ended around 260 Myr^{3,15}.

Allowing for uncertainty, either graph might account for known climatic trends. I suggest that Fig. 1a points to a new instability of the climate, underlying the widely-accepted instability of Fig. 1b.

The new instability would work as follows. When drift concentrates land in the polar caps, the tropics get wetter. Albedo contrast produces slight polar cooling and substantial warming of the tropics. On balance, the steeper polewards temperature gradient is overshadowed by the increase in world average temperature. There may be periods, such as the early Cretaceous¹⁹, of 'stable cold' limited regionally and seasonally, but global warmth is the rule, exemplified by the Mesozoic as a whole. When landmasses drift into the tropics, the caps get wetter and slightly warmer while the tropics cool substantially. Global cooling overshadows the reduced temperature gradient, and cold periods are unstable: they are more likely to intensify by the persistence of fallen snow and, for snow to persist, dry land must be suitably placed in high latitudes. This second source of instability implies that land over the poles is necessary but not sufficient for glaciation. Figure 1a and b are thus not inconsistent. Figure 1a yields a stronger necessary condition: land concentrated in the tropics as well as near the poles. By implying watery mid-latitudes, this supports ideas of the circulation required to nourish polar glaciers⁴.

The significance of these landmass motions can be assessed with a simple climatic model. For each 10° belt the absorbed solar radiation is

$$Q_a = (1 - \alpha) m Q_s \quad (1)$$

where $Q_s \propto S/4$ is the incident radiation without the atmosphere²⁰, with the solar constant $S = 1,361 \text{ W m}^{-2}$. $m = Q_*/Q_s = 0.59$ is atmospheric transparency, fixed at the modern world average²¹. The surface albedo α is a mean of land and water albedos, α_L and α_w , weighted by area.

α_w depends on latitude as follows:

Latitude	0–50	50–60	60–70	70–80	80–90
Albedo	5	6	8	10	12

with weight given to the work of Sivkov²² and Grishchenko⁹ and to the knowledge that q_d is large under clouds²³. α_L was chosen to be constant at 15, close to modern albedos for temperate forests^{24,25}, marshland²⁴, equatorial rain forest⁸, wet-season savanna⁸ and tundra in summer²⁴. Desert²⁴, at 17–29, is the only large terrain type brighter than 15. A constant α_L may seem questionable, but the argument hinges solely on a decrease in $(\alpha_L - \alpha_w)$ with latitude. The claim is one of logic, not of numerical realism.

Placement errors affect the calculated radiation, whether or not they sum to zero, but this uncertainty is much smaller than the observed range of 0.41 W m^{-2} (Fig. 1c). A rough numerical idea of the corresponding range of temperature $\Delta \bar{T}$ can be obtained from the expression²⁶ $\Delta \bar{T} = k (\Delta S/S)$, where ΔS is a prescribed change in the solar constant and $k \approx 140$ for several albedo–feedback models. From equation (1), making ΔS an independent variable, $\Delta \bar{T} \propto \Delta Q_a \approx 0.3 \text{ K}$ for the past 240 Myr. Continental drift seems a modest governor of climatic change. However, the calculations assume no snow, which is certainly unrealistic for the Cenozoic. If there is an instability, actual insolation must deviate progressively from Fig. 1c through the past 60 Myr. The absorption calculated for the present gives, when compared with the observed²¹ 171.5 W m^{-2} , a tempera-

ture difference of 9.5 K, agreeing well with the broad average difference inferred between the present and the Mesozoic²⁷.

When the drift sequences of the Palaeozoic are better known, more extensive tests of this work will be possible. It may have defined a stronger necessary condition for glaciation, but not its sufficiency. The idea is more firmly grounded than galactic hypotheses²⁸; if there is a galactic mechanism, it may modulate known tectonic processes to bring about glacial periods.

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Hidden genetic variability in two populations of a marine mussel

ELECTROPHORESIS detects only about one third of the genetic variation present in a natural population, despite its wide application¹. A combination of electrophoretic and heat denaturation techniques has revealed considerable molecular variation within single electrophoretic classes of enzymes. For example, Bernstein *et al.*² have found 1.74 more alleles at the xanthine dehydrogenase locus in the *Drosophila virilis* group than had been detected by electrophoresis alone. Further such evidence^{3–11} has concentrated on species of *Drosophila*. I report here a study of the marine mussel *Guekensia demissa* (formerly *Modiolus demissus*) which shows that in two geographically separated populations experiencing different temperature regimes, there is an apparent correlation between the distribution of thermosensitive alleles at the phosphoglucosylase (*Pgm*) locus and environmental temperature.

Samples of *G. demissa* were collected from a salt marsh at Beaufort, North Carolina (35° N) and from the intertidal region of a beach at Stony Brook, Long Island, New York (41° N). Water temperatures in the Beaufort region can reach a summer maximum of 33 °C while in Long Island Sound they rarely reach 28 °C. Air temperatures in both regions are correspondingly higher¹².

Table 1 Distribution of genotypes and allele frequencies at the *Pgm* locus in two populations of *Guekensia demissa* from Beaufort and Stony Brook

	N*	AA	BB	Genotypes					Allele frequency				χ^2
				CC	AB	AC	BC	BD	A	B	C	D	
Beaufort	103	9	22	5	29	17	20	1	0.31	0.46	0.23	0.01	0.61NS
Stony Brook	51	4	9	6	9	6	17	—	0.23	0.43	0.34	0.0	0.49NS

* Number of individuals analysed.

 χ^2 , Goodness-of-fit of observed to expected frequencies from Hardy-Weinberg equilibrium.NS, $P > 0.05$.**Table 2** Distribution of genotypes and allele frequencies at the *Pgm* locus in two subsamples of *Guekensia demissa* from Beaufort and Stony Brook retained for heat stability studies

	N	AA	BB	Genotype					Allele frequency				χ^2
				CC	AB	AC	BC	BD	A	B	C	D	
Beaufort	39	5	8	3	10	6	6	1	0.34	0.42	0.23	0.01	1.43NS
Stony Brook	40	5	7	6	7	3	12	—	0.25	0.41	0.34	0.0	4.47NS

In *G. demissa* *Pgm* is coded at a single locus, with three common alleles *Pgm*^A, *Pgm*^B and *Pgm*^C present in both populations, and a rare allele *Pgm*^D detected only in the Beaufort sample. Two procedures were initially used to evaluate the thermostability of these *Pgm* alleles. First, two aliquots of a homogenate of adductor muscle from a single individual were incubated at two temperatures, followed by electrophoresis and staining; second, slices of a starch gel were incubated at two temperatures after electrophoresis of a homogenate of adductor muscle from a single individual, followed by the addition of *Pgm* stain. Temperature and duration of incubation were decided empirically. Heat stability studies were performed using the first procedure in preference to the second because conditions could be controlled more easily.

Table 1 presents allele frequency data for the two samples of mussels. The samples were similar in allele frequency and no significant departures from Hardy-Weinberg expectations were observed. No significant differences in either genotypic ($P = 0.20$) or allelic ($P = 0.07$) proportions were observed when the two samples were compared. Smaller numbers of individuals within each of these samples were used for thermostability studies and Table 2 gives allele frequency data for these subsamples. Allele frequencies were similar to those observed in Table 1, with no significant departures from Hardy-Weinberg equilibrium. In addition, no significant differences in either genotypic ($P = 0.52$) or allelic ($P = 0.28$) proportions were observed between the two smaller samples.

A *Pgm* band was classified as being either heat-resistant (in this case *Pgm* activity present after 15 min at 60 °C) or heat-sensitive (no *Pgm* activity after 15 min at 60 °C). Two kinds of

homozygotes were observed: those in which the *Pgm* band was either heat-sensitive or heat-resistant. Four kinds of heterozygotes were observed: those in which both *Pgm* bands were heat-sensitive or heat-resistant and those in which one or other of the *Pgm* bands was heat-sensitive (Fig. 1). This suggests that heat-sensitivity of each *Pgm* electrophoretic band resides at the level of the *Pgm* structural gene. Table 3 presents allele frequency data for the two populations when electrophoretic and heat-sensitivity determinations were combined. Both heat-sensitive and heat-resistant forms of the alleles *Pgm*^A, *Pgm*^B and *Pgm*^C were observed in the two samples of mussels. In the Beaufort sample, where *Pgm*^D was observed at low frequency, only the heat-sensitive form of *Pgm*^D was detected. This newly discovered variation increases heterozygosity values (calculated from observed allelic frequencies) for this locus from 0.66 to 0.73 in the Beaufort sample and from 0.65 to 0.79 in the Stony Brook sample. Sixty-eight of the 78 alleles investigated in the Beaufort sample were heat-resistant and in the Stony Brook sample 56 of the 80 alleles investigated were heat-resistant. Analysis was performed on these numbers using a 2×2 contingency χ^2 test and the distribution of heat-sensitive and heat-resistant *Pgm* alleles was found to be significantly different in the two populations ($0.01 > P > 0.001$). The frequency of heat-sensitive forms of the three major alleles was consistently higher (particularly for *Pgm*^B) in the sample of mussels from the cooler waters of Long Island Sound. Also, the frequency of individuals homozygous for resistant alleles was significantly greater ($0.05 > P > 0.02$) in Beaufort (31 out of 39 individuals) than in Stony Brook (23 out of 40 individuals) and the frequency of individuals homozygous for sensitive alleles was greater in Stony

Table 3 Distribution of genotypes and allele frequencies at the *Pgm* locus for two populations of *Guekensia demissa* when electrophoretic and heat sensitivity determinations are combined

	Genotypes		A ^s	A ^r	B ^s	B ^r	C ^s	Allele frequency		D ^r	χ^2
								C ^r	D ^s		
Beaufort <i>n</i> = 39	A ^r A ^r	5	0.03	0.31	0.08	0.35	0.01	0.22	0.01	0.0	5.48NS
	B ^r B ^r	7									
	C ^r C ^r	3									
	B ^s B ^s	1									
	A ^s B ^r	7									
	A ^s B ^s	1									
	A ^s B ^r	1									
	A ^s B ^s	1									
Stony Brook <i>n</i> = 40	A ^r A ^r	4	0.04	0.21	0.19	0.23	0.08	0.26	0.0	0.0	3.25NS
	B ^r B ^r	4									
	C ^r C ^r	5									
	A ^s A ^s	1									
	B ^s B ^s	3									
	C ^s C ^s	1									
	A ^r B ^r	4									
	B ^s C ^r	5									

Superscript s and r correspond to heat-sensitive and heat-resistant forms of *Pgm* alleles.

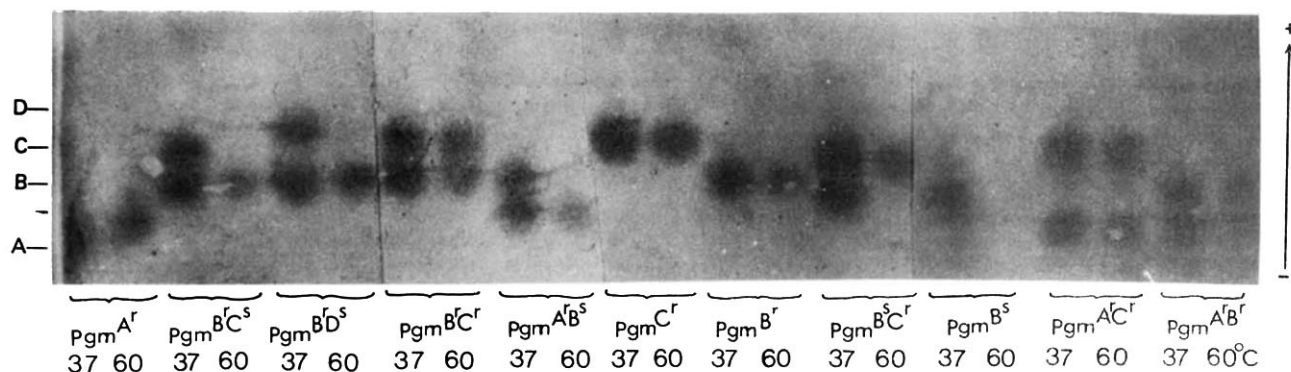


Fig. 1 Heat sensitivity of *Pgm* alleles in *Guekensia demissa*. (Results of two gels from the same electrophoretic run; gel papers were divided and transposed to eliminate repetitive patterns.) The posterior adductor muscle was excised from living individuals and homogenised manually in approximately two volumes of 50 mM Tris-HCl buffer, pH 7.8 (1:1 w/v). Homogenates were centrifuged at 3,500 r.p.m. for 15 min at 4 °C. The supernatant solution was divided into four aliquots, two of which were incubated in a water bath for 15 min, one at 37 °C and the other at 60 °C. The remaining two aliquots were retained for replication; hence each individual was classified on the basis of two replicated experiments. Electrophoresis was performed at 2 °C in horizontal starch gel (Electrostarch) using the Tris-EDTA-maleic buffer system, pH 7.4, of Spencer *et al.*¹³. Gels were run for 14–16 h at 6.5 V cm⁻¹. Sliced gels were stained for *Pgm* using the paper overlay method described by Scopes¹⁴. The terminology used for allozymes of *Pgm* is as follows: alleles are numbered A to D in order of increasing anodal mobility. For comparative purposes individuals from both populations were run on the same gel.

Brook (7 out of 40 individuals) than in Beaufort (2 out of 39), although this difference is not significant at the 5% level (0.10 > P > 0.05).

In spite of increasing experimental evidence for hidden genetic variability within electrophoretic allozyme classes—at least for species of *Drosophila*—there have been no reports to date on the possible biological significance of this new-found structural variability. Some workers^{2,5} commenting on the random distribution patterns of some of the heat-sensitive allozymes of xanthine dehydrogenase and octanol dehydrogenase in the *Drosophila virilis* group concluded that such patterns support the neutral and not the selection hypothesis of genetic variability in natural populations. It is possible that the apparent non-random distribution of thermosensitive alleles in the mussel populations investigated in the present study is being maintained through selective forces acting at the *Pgm* locus. Trippa *et al.*⁹ have suggested 'that selective values of allelic proteins with different thermosensitivities might in fact be affected by differences in the habitat', perhaps in this case temperature, an important component of an organism's environment. However, before firm conclusions can be drawn regarding the adaptive role of hidden genetic variation within populations of *G. demissa*, detailed biochemical investigations of the *Pgm* system are necessary. Also, breeding data—at present unavailable—would clarify whether the property of thermosensitivity is associated with the *Pgm* locus itself or whether it is associated with an alternative polymorphic locus.

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Mutation at H-2K locus influences susceptibility to autoimmune thyroiditis

GENES in the major histocompatibility complex (MHC) have been found to be associated with the development of autoimmune diseases, including spontaneous diseases in man^{1,2} and spontaneous³ or experimentally induced autoimmunity in animals⁴. Experimental autoimmune thyroiditis (EAT) can be induced in susceptible strains of mice, such as those with the MHC H-2^k haplotype, by injecting them with mouse thyroglobulin together with complete Freund's adjuvant⁵. In contrast, injection of mice homozygous for the H-2^b haplotype fails to induce the mononuclear cell infiltration of the thyroid gland characteristic of EAT. We report here that susceptibility to induction of EAT results from an apparent point mutation which evidently occurred at the H-2K locus of the resistant H-2^b haplotype. This indicates that the H-2K glycoprotein can serve to regulate the autoimmune response to thyroglobulin.

Table 1 shows the results of injecting mouse thyroid extract in adjuvant into mice of strains C3H/eb (H-2^k), C57BL/6 (H-2^b) or its HZ1 mutant (B6.C-H-2^{ba})^{6,7}. C3H/eb mice demonstrated the response of high-responder H-2^k strains with about 80% incidence of development of EAT. Only about 20% of C57BL/6 mice developed lesions of EAT, as expected for low responder mice of H-2^b strains^{5,8}. However, the HZ1 (H-2^{ba}) mutant showed an incidence of EAT (79%) comparable with that of high-responder H-2^k mice. Hence, the HZ1 mutation led to susceptibility to EAT in mice whose original genome coded for resistance.

The immunogen used in these experiments was thyroid extract obtained from C3H/eb mice. Mouse strains differ in the immunogenicity of their thyroglobulin⁹ and we have found that thyroid extract from C3H/eb mice is strongly immunogenic whereas that of both C57BL/6 and HZ1 mice is poorly immunogenic for either high- or low-responder strains of mice (in preparation). These findings suggest that the increased

Table 1 Susceptibility to EAT of the HZ1 mutant (H-2^{ba}), and H-2^b low- and H-2^k high-responder strains of mice

Mouse strain	H-2 haplotype	Injection of thyroid extract plus adjuvant	Incidence of EAT	Antibody titre (log ₂)
C3H/eb	k	Yes	82% (46/56)	5.0
C57BL/6	b	Yes	20% (10/49)	5.0
HZ1	ba	Yes	79% (11/14)	7.0
		Adjuvant alone	0 (0/5)	<1.0

Mice were injected twice subcutaneously at 7-d intervals with thyroid extract emulsified in complete Freund's adjuvant, as described by Twarog and Rose²¹. The extract was prepared from thyroid glands of C3H/eb mice. Each mouse received the equivalent of one thyroid gland. The adjuvant²¹ was prepared by adding 7 mg ml⁻¹ of *Mycobacterium tuberculosis* H37Ra (Difco) to incomplete Freund's adjuvant (Difco). The animals were killed 5 weeks after the second injection and histological sections of the thyroid glands were examined in a blind fashion by two independent observers. A thyroid gland was considered to be positive for EAT if it showed at least one unequivocal focal infiltrate of mononuclear cells⁹. Antibodies to purified thyroglobulin, prepared as described by Tomazic and Rose⁹, were measured in the pooled serum of mice in each group, using a haemagglutination of formalinised tanned sheep red blood cells²² coated with thyroglobulin. The results represent three separate experiments. Difference in incidence of EAT between C57BL/6 and HZ1 was highly significant by the χ^2 test ($P < 0.0003$).

incidence of EAT in the HZ1 mutant mice was not due to a change in the immunogenicity of their thyroglobulin.

Note that all groups developed antibodies to purified thyroglobulin after injection with thyroid extract in adjuvant. This confirms the observation that mice develop specific antibodies to determinants of thyroglobulin after injection with extract, whether or not they are genetically susceptible to histological EAT¹⁰.

The HZ1 mutant has been studied in several laboratories^{7,11-14} and it is generally concluded that a point mutation occurred at the H-2K locus of the C57BL/6 genome. This conclusion is supported by the finding of differences in the peptide map of the H-2K glycoprotein of the HZ1 mutant, compared with the wild-type C57BL/6 strain¹⁵. This molecular difference would seem to account for the mutual T-cell reactivity of C57BL/6 and HZ1 (refs 13, 16). However, the HZ1 mutant seems to preserve other determinants on the H-2K molecule which are identical to those of the H-2K^b wild type^{7,12}.

There is no evidence of any differences between C57BL/6 and HZ1 in the I region to the right of the H-2K locus, as the immune response (Ir) genes which functionally define the I region are the same in the mutant and wild-type mice¹⁷. Thus, it seems very likely that the only genetic differences between the wild-type C57BL/6 and the HZ1 mutant mouse are at the H-2K locus of the MHC⁷. Hence, our results indicate that the H-2K glycoprotein can regulate the pathological expression of EAT following an autoimmune response to thyroglobulin. This implies a major role for cytotoxic T cells in EAT. Furthermore, the limited portion of the H-2K^b gene in which the mutation occurred, the Z1 locus⁶, seems to be critical in determining susceptibility to EAT.

Previously, H-2K or H-2D gene products have been observed to restrict the cytotoxic effects of T lymphocytes against target cells infected with certain viruses¹⁸. T lymphocytes obtained from mice immunised against viruses will often kill virus-infected target cells only when the target cells and the lymphocytes have H-2K or H-2D genes in common. Note that HZ1 and C57BL/6 differ in the specificity of virus-H-2K-associated cytotoxicity¹³. It is thought that associative recognition by T lymphocytes of viral antigens together with MHC gene products is based on the molecular association of viruses with MHC gene products at the surface of the infected cell^{19,20}. It remains to be determined whether susceptibility to EAT also

involves physical association between H-2K glycoprotein and thyroglobulin on the surface of thyroid epithelial cells. Such an association could occur if MHC gene products served to control movement of macromolecules across cell membranes.

The different susceptibilities to induction of EAT of the C57BL/6 strain and its HZ1 mutant suggest that these mice may be helpful in determining how MHC gene products regulate immune responses. Any differences between the mice in the handling or presentation of thyroglobulin by macrophages, lymphocytes or other cells could be related to the specific modification of the H-2K glycoprotein. Furthermore, isolation of purified H-2K glycoproteins may allow study of the interaction of these molecules with specific determinants of thyroglobulin.

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A novel subset of antigenic cells triggers B-cell responses to MHC antigens

THE exceptional immunogenicity of major histocompatibility complex (MHC) antigens is well known but poorly understood. As there is evidence that MHC products are intimately involved in the presentation of antigen to T cells^{1,2} it seems reasonable to expect that antigenic variants of these 'presenting structures' should be very immunogenic for T cells. However, because MHC antigens on non-nucleated cells and in subcellular forms are weak T-cell immunogens and may even function as tolerogens³, it has been postulated that an additional signal from living antigenic cells is needed to stimulate the immune response⁴. The response of B cells to MHC antigens is far less well understood but again an additional signal from antigenic cells seems to be needed in some primary responses, as antigenic cell fragments are not immunogenic⁵. We report here that in studies of primary antibody responses to H-2-incompatible cells, immunogenicity is almost completely restricted to a small, novel subpopulation of cells found in the spleen and bone

Table 1 For induction of primary anti-H-2 PFC responses conventional T-cell competence in donors and recipients is not required

Experiment	Recipient strain	Donor strain	Dose of donor spleen cells	PFC per spleen Mean (s.e.m.)
1	CBA	B10 nu/+ and +/+	2 × 10 ⁶	936 (155)
	CBA	B10 nu/nu	2 × 10 ⁶	2,136 (139)
2	nu/+ and +/+	B10 nu/nu	10 ⁶	1,800 (278)
	nu/nu	B10 nu/nu	10 ⁶	2,730 (449)
3	nu/+ and +/+	BALB/c	10 ⁷	14,866 (4,050)
	nu/nu	BALB/c	10 ⁷	8,370 (2,773)
4a	nu/nu	BALB/c nu/nu	2.3 × 10 ⁶	40,577 (8,452)
	nu/+ and +/+	BALB/c nu/nu	2.3 × 10 ⁶	10,450 (3,215)
4b	nu/nu	BALB/c nu/+ and +/+	2.3 × 10 ⁶	26,717 (6,331)
	nu/+ and +/+	BALB/c nu/+ and +/+	2.3 × 10 ⁶	21,008 (4,944)

Anti-H-2^b responses were determined in experiments 1 and 2 and anti-H-2^d responses in experiments 3 and 4. PFC were assayed by the technique developed by Fuji *et al.*⁶ and modified from our method for assay of anti-Thy-1 PFC¹². Briefly, 50 µl of appropriate concentrations of spleen cells in Eagle's modified minimal essential medium (MEM) containing 10% heat-inactivated newborn calf serum (MEM+CA₁₀) were mixed with 20 µl of 20–40% (packed volume) of appropriate target cells. The suspension was warmed to 37 °C and 180 µl of 1% agarose in MEM-CA₁₀, kept at 45–48 °C, was added immediately before pouring on to 30-mm Petri dishes. These plates were incubated at 37 °C in a humidified 10% CO₂ atmosphere for 3.5 h, and then an anti-mouse Ig, rabbit antiserum (0.5 ml at 1:10) was added. Following a further incubation at 37 °C for 30 min 1.0 ml of selected rabbit complement at 1:15 dilution (in 10% hypotonic MEM-CA₁₀ containing 3 mM barbituric acid) was added. After 45 min incubation with complement, these plates were stained with 0.2–0.3% of Trypan blue in phosphate-buffered saline for 15 min. The numbers of plaques were counted with the aid of a dissecting microscope. Almost all PFC detected by this technique were IgM-secreting cells, as addition of a specific anti-µ serum (20 µl of 1:60 dilute antiserum) into the mixture of spleen cells and target cells before incubation inhibited the development of plaques. Primary PFC responses assayed on day 5 are shown.

marrow. Furthermore, as the response we have studied is independent of T cells, these stimulating cells seem to be unique (perhaps specialised?) in their ability to present surface-borne antigen and the additional signal to responding B cells.

The experimental system was developed from the anti-H-2 direct plaque-forming cell (PFC) assay of Fuji *et al.* in which specificity for H-2K and H-2D region antigens has been established and other parameters of response have been defined^{6,7}; these findings have since been confirmed in our laboratory (in preparation). Primary responses to H-2 antigens were induced by intraperitoneal injection of spleen cells into appropriate recipients. Spleens of responding mice were removed after 5 days, at the peak of response, and their content of PFC determined in the plaque assay using the lymphoma lines L1210(H-2^d) or EL-4G⁻(H-2^b) as targets.

To examine the role of T cells in the response, normal or T-cell deficient mice were immunised with antigenic spleen cells from normal or T-cell deficient donors. The response of CBA mice to spleen cells from C57BL/10 or C57BL/10 nu/nu donors (H-2^b) shows that T-deficient cell populations may in fact provoke stronger responses than C57BL/10 nu/+ control donor mice, which have a normal T-cell compartment (Table 1). Thus, T cells in the inoculum are apparently unnecessary for the induction of primary PFC in these conditions. Using outbred nu/nu and littermate +/+ and nu/+ recipients in combination with C57BL/10 nu/nu donors it is apparent (Table 1) that T-cell deficient recipients respond equally well or better than their controls to C57BL/10 nu/nu donors. Similarly, the response to H-2^d antigens is also substantially independent of T cells in the donor or in responding mice (Table 1, experiments 3, 4). Thus, conventional T-cell functions seem to have no role in the primary PFC response to H-2 antigens.

These results significantly strengthen previous evidence that primary responses to MHC antigens of mice (and rats) are mainly independent of conventional T-cell activity^{8,9}. It is, however, unexpected that glycoproteins such as MHC antigens

should induce substantial T-cell independent responses. First, their physical properties are at marked variance with properties common to other T-cell independent antigens, such as a polymeric molecular structure and slow catabolism *in vivo*¹⁰. Second, and in contrast to H-2, we have shown that the primary PFC response to another glycoprotein surface antigen, Thy-1, is dependent on T-cell mediated helper effects produced by other surface antigens coded elsewhere in the donor genome^{11–13}. Taken together, these apparently contradictory observations suggest that the molecular and antigenic properties which determine the immunogenicity of cell-surface antigens cannot be predicted from present knowledge and are likely to be quite different from those for molecular antigens in solution.

In further experiments, to be reported in detail elsewhere, we found that some lymphoid tissues were markedly more immunogenic than others (for example, spleen cells are approximately 20 times more active at stimulating PFC than lymph node cells, and unfractionated peritoneal cells are virtually inactive). However, the density of H-2 antigens as determined by absorption tests was indistinguishable in the different cell populations. We therefore postulated that immunogenicity for primary PFC may not be related simply to the amount of H-2 antigen on the donor cell inoculum but could reside in an immunogenic subpopulation of cells which is present in higher frequency in the spleen. To examine this possibility donor spleen cells were fractionated with respect to (1) adherence to nylon

Table 2 Characterisation of spleen cells immunogenic for primary anti-H-2 PFC

Experiment (recipient strain)	Donor pre-treatment	Fractionation		Cell recovery (%)	Dose of viable cells	PFC per spleen
		Adherence	Density			
1. (CBA)		Unseparated		100	2 × 10 ⁶	2,136
		Adherent		31 (55)	2 × 10 ⁶	900
		Non-adherent		22 (45)	2 × 10 ⁶	5,028
2. (CBA)		Unseparated			1 × 10 ⁶	1,356
		Non-adherent			1 × 10 ⁶	1,548
		Non-adherent	Low†		5 × 10 ⁵	0
		Non-adherent	Low†		5 × 10 ⁶	0
		Non-adherent	High†		5 × 10 ⁵	1,608
3. (CBA)		Non-adherent			1 × 10 ⁵	0
		Non-adherent			5 × 10 ⁵	348*
		Non-adherent	Low	62 (85)	5 × 10 ⁵	0
		Non-adherent	Low		5 × 10 ⁶	0
		Non-adherent	High	11 (15)	1 × 10 ⁵	120*
		Non-adherent	High		5 × 10 ⁵	1,425
4. (C57 BL/10)		Adherent			1 × 10 ⁵	225*
		Non-adherent	Low		1 × 10 ⁵	0
		Non-adherent	Low		2 × 10 ⁶	20*
		Non-adherent	High		1 × 10 ⁵	32,364
5. (CBA)		Normal spleen		100	1 × 10 ⁵	75*
		Normal spleen			1 × 10 ⁶	9,945
		Normal spleen			1 × 10 ⁷	7,704
	Irradiation	Unseparated		8	5 × 10 ⁴	15*
	Irradiation	Unseparated			1 × 10 ⁵	7,632
	Irradiation	Adherent			1 × 10 ⁵	1,020
	Irradiation	Non-adherent	Low		1 × 10 ⁵	0
	Irradiation	Non-adherent	High		5 × 10 ³	315
	Irradiation	Non-adherent	High		5 × 10 ⁴	13,812

The results show that the immunogenicity belongs to a small subset of cells which is relatively non-adherent to nylon wool, of unusually high density, and resistant to irradiation administered *in vivo*. Donor BALB/c mice were irradiated at 900 R from a ⁶⁰Co source 20 h before removal of spleens. For fractionation, 1 ml of an appropriate concentration (5 × 10⁷ ml⁻¹) of spleen cells from normal or irradiated BALB/c mice in MEM containing 5% fetal calf serum (MEM-F₂) were loaded on nylon wool columns each of 0.6 g in a 6-ml volume. Nylon-non-adherent cells were eluted after incubation of the columns at 37 °C for 1 h by the procedure of Julius *et al.*²². Nylon-adherent cells were also collected after thoroughly washing the columns according to the method of Trizio and Cudkowicz²³. Five ml of the suspension of nylon-non-adherent cells (<10⁷ p/ml⁻¹) were layered on 3 ml of Ficoll-Hypaque solution (ρ = 1.082) and centrifuged at 3,000 r.p.m. for 20 min at 20°C. The pellet fraction (high-density fraction) and interface fraction (low-density fraction) were collected and washed three times with MEM at 150 g for 8 min. Day 5 primary PFC responses are shown. Each value represents the mean of five mice. The standard error of each value is less than 25%, except the values marked with an asterisk (≥25%). Donor cells for experiment 4 were irradiated *in vitro* at 2,000 R before injection. Cell recovery is given as the percentage of recovery of cells in each fraction to overall recovery of cells after fractionation.

† Treated with 0.83 M ammonium chloride to lyse erythrocytes before injection.

Table 3 Ability of spleen cell fractions to stimulate PFC to H-2 antigens does not correlate with their stimulatory ability in MLC

Cell fraction	Buoyant density	MLC		PFC per spleen
		c.p.m.	Index	
Nylon-adherence				
Original		65,112	5.3	
Adherent	—	45,748	3.7	225 (61)
Non-adherent	—	29,040	2.3	
Non-adherent	Low	43,972	3.6	0 (0)
Non-adherent	High	7,854	-0.6	32,364 (6,990)
(Syngeneic control)		12,264	1.0	

For MLC, 6×10^5 CBA spleen cells in 0.1 ml of RPMI medium containing 10 mM HEPES, 5×10^{-5} M 2-mercaptoethanol and 5% heat-inactivated fetal calf serum were mixed with 6×10^5 CBA spleen cells (syngeneic control) or BALB/c spleen cells which had been fractionated and irradiated at 2,000 R. They were incubated in a humidified, 10% CO₂ atmosphere at 37 °C for 5 d, and then pulsed with 1 μ Ci of [¹²⁵I]5-iodo-2'-deoxyuridine. After 4 h incubation the cultures were collected on filter paper for counting. MLC results are the mean of three to four cultures. PFC results are day 5 primary anti-H-2 PFC responses of B10 mice injected with 10^5 cells. This is part of experiment 4 in Table 2. Values represent mean PFC (s.e.m.).

wool, (2) buoyant density and (3) resistance to γ -irradiation administered *in vivo* 1 day before the donors were killed. It is clear from results using nylon wool columns (Table 2, experiments 1, 2, 4, 5, and Table 3) that on comparing inocula of viable cells, the immunogenic cells are enriched in the non-adherent fraction and significantly depleted in the adherent fraction. Subsequent fractionation of the non-adherent cells on Ficoll-Hypaque (density 1.082 g cm⁻³) showed that immunogenic cells are enriched in the dense fraction (the pellet) and virtually absent in the lymphoid cell-rich, interface buoyant fraction (Table 2, experiments 2-5, and Table 3).

The radiosensitivity of the immunogenic cell fraction was assessed by irradiation of donors with 900 R from a ⁶⁰Co source 20 h before removal of their spleens. It is evident (Table 2, experiment 5) that spleen cells from irradiated donors are considerably more immunogenic than similar numbers of normal spleen cells. Further fractionation of this irradiated population of nylon wool columns resulted in decreased immunogenicity in the adherent cell fraction, as before. The non-adherent fraction was separated on Ficoll-Hypaque and showed, also as before, no immunogenicity of the interface cells but very high immunogenicity of the dense fraction. The factor of enrichment for the subpopulation of immunogenic cells with the three procedures in combination is estimated to be 50- to 70-fold. The data presented above, together with the unusual tissue distribution of the immunogenic cells, their presence in nude mice, the results of the fractionation procedures and some unpublished results, make it unlikely that the immunogenic cells are conventional T, B or macrophage-like cells. Furthermore, cells of the granulocytic series which are usually enriched in high-density fractions are highly radiosensitive and adherent to nylon, as confirmed by microscopic observation.

Recent evidence indicates that specialised accessory cells are highly efficient in the presentation of antigen to T cells¹⁴, and these cells are the most active in stimulating allogenic responses in mixed leukocyte cultures (MLC)¹⁵. As the MHC antigens on the non-adherent, dense cells stimulated B cells to produce PFC in our experiments, we also examined the ability of these cells to stimulate T cells in MLC¹⁶. As shown in Table 3 no correlation was observed between cell fractions which stimulated in MLC and those which stimulated PFC in the same experiment. In fact, the non-adherent high-density fraction, which is by far the most effective in inducing PFC, reproducibly induced little or no MLR. This result, taken with the other physical parameters considered above, seems to distinguish the novel PFC-stimulating fraction from the cells considered to be functionally specialised for antigen presentation to T cells^{14,15,17}.

The normal role of these B-stimulating cells in the immune system is unknown but their unusual properties should facilitate investigation. However, *in vitro* studies show that accessory cells or antigen-presenting cells are involved in B-cell triggering for

T-cell dependent¹⁸ and possibly some T-cell independent responses¹⁹⁻²¹. Such cells may be identical to the antigen-presenting cells for T-cell responses or may comprise a new subpopulation of cells. The novel subset of cells reported here to be exceptionally immunogenic for B cells may have a role in antigen presentation and B-cell triggering.

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CFU-S in individual erythroid colonies derived *in vitro* from adult mouse marrow

It is generally accepted that in the haematopoietic system there is a hierarchy of pluripotent and committed progenitor cell types. Representative committed progenitors from all the myeloid and lymphoid pathways can be detected by *in vitro* colony assays¹. This methodology has been extended to include multipotent haematopoietic progenitors²⁻⁵, and it has since been shown that at least one such multipotent cell type, the progenitor of macroscopic erythroid-megakaryocyte bursts, undergoes self-renewal during colony formation *in vitro*⁵. We now report that cells capable of macroscopic spleen colony formation (CFU-S)⁶ are present in macroscopic bursts and that on average two to three and up to five self-renewal divisions of CFU-S can occur during the first 9 d of burst growth. These findings raise the possibility that clonal analysis techniques⁷ may be combined with cell culture manipulations to investigate how the choice between self-renewal and progressive differentiation is determined.

Macroscopic bursts were generated in standard methylcellulose cultures containing 2.5 units ml⁻¹ erythropoietin (Connaught, Step III) and pokeweed mitogen-stimulated spleen cell conditioned medium (final concentration 0.3%) as previously described⁵. These conditions are optimal for macroscopic burst formation and give a linear relationship between

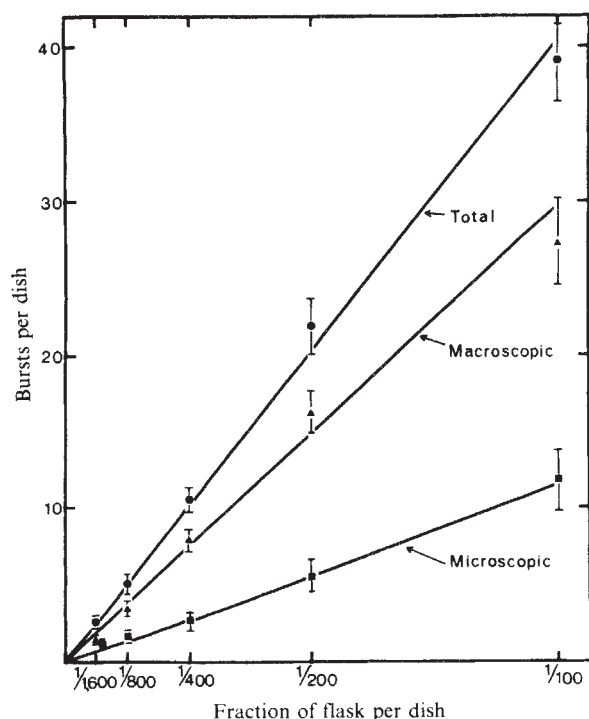


Fig. 1 Burst formation as a function of the number of cells plated. Cells were pooled from three replicate 2-week-old flask cultures maintained and assayed as described in the text. The number of cells in each assay dish is expressed as a fraction of the total number of non-adherent cells collected per flask. In this experiment 1/100th of a flask contained 6.4×10^4 cells. Values shown represent the mean count ± 1 s.e.m. for a minimum of four assay replicates.

burst number and cell concentration (Fig. 1). They are inadequate for significant granulocyte colony formation. The cells used for plating were from the non-adherent cell fraction of 2-week-old flask cultures prepared using adult B6C3F₁ mouse marrow cells as previously described^{8,9}. Although cells capable of macroscopic burst formation are present in fresh as well as cultured adult marrow, their incidence in fresh marrow is much lower (only 5–10 per 10^5 nucleated cells) and they constitute less than one-third of the total late-maturing burst progenitor population⁵. In the conditions of flask culture used here, there is a preferential proliferation and survival of macroscopic burst progenitors so that by 2 weeks their concentration reaches 30–50 per 10^5 nucleated cells and they constitute the predominant erythropoietic cell type^{5,8}. Thus, by plating 2.5×10^4 flask cultured cells (that is, 1/200th of a flask, washed and diluted in α -medium plus 2% fetal calf serum) 10–20 well isolated macroscopic bursts were obtained in each assay dish (Fig. 1), and the problem of contaminating background colonies derived from either granulopoietic progenitors (CFU-C) or more mature erythropoietic progenitors (day 14 BFU-E micro, day 3 BFU-E, CFU-E) was also eliminated. After 9 d of incubation, macroscopic bursts could be readily identified as red spots visible to the unaided eye (Fig. 2a). Such bursts were picked and assayed for CFU-S (see Table 1 legend for details). Macroscopic spleen colony counts were carried out on day 9 (Fig. 2b).

Of 25 macroscopic bursts assayed individually, 11 (44%) produced spleen colonies (Table 1a). On average each produced one spleen colony although values of up to four were obtained. The incidence of approximately one CFU-S per macroscopic burst was confirmed when suspensions consisting of four or eight pooled macroscopic bursts were assayed, and the proportion of pools that were positive increased to 78% and 87%, respectively. The wide distribution of values obtained for different pools of four and even eight bursts also confirms the heterogeneity seen when individual bursts were assayed. Presumably, some of the macroscopic bursts contained at least four times the overall average number of CFU-S. Using an estimate of 0.15 for

the seeding fraction (f)¹⁰, the total CFU-S per macroscopic burst was on average 6.7 and must have reached at least 27 in some bursts. This would indicate an average of two to three, and up to five self-renewing cell divisions.

Spleen colony formation was not associated with a cell type that occurred randomly throughout the burst assay culture, for aliquots of 'background' culture equivalent to three times the culture volume contained in a pool of eight macroscopic bursts did not produce spleen colonies (Table 1a). Irradiation of the injected cells also eliminated spleen colony formation (Table 1b). In addition, karyotype analysis of metaphases in female recipient spleens removed 10 d after irradiation and injection with male macroscopic bursts showed 33 of 37 metaphases to be of male, that is, burst cell, origin (of the remaining four metaphases, three were scored as probable males and one as female). For this analysis three spleens with visible surface colonies were made into single cell suspensions 1–2 h after intraperitoneal injection of 50 μ g colcemid. Metaphase spreads were C or trypsin G-banded and a sample of technically suitable metaphases photographed and evaluated by P.J. and F.D. for 40 chromosomes and the Y chromosome¹¹. These results indicate that the spleen colonies obtained resulted from proliferation of cells originally present in macroscopic bursts.

Previous experiments with macroscopic bursts have suggested a close relationship between their progenitors and CFU-S⁵. The present findings provide direct evidence for overlap between the cell populations detected by these two assays. Further experiments will be required for full characterisation of the *in vivo*

Table 1 Number of spleen colonies produced by samplings from macroscopic burst assays

a Macroscopic burst cells vs surrounding culture medium (background)				
	1 Macro burst per recipient	4 Macro bursts per recipient	8 Macro bursts per recipient	Background
Exp. 1	2, 0, 4, 2, 0	13, 1, 0, 1, 17	4, 13, 1, 2, 3	0, 0, 0, 1
Exp. 2	2, 0, 0, 1, 1	3, 1, 0, 6, 0 0, 3, 4, 5	12, 2, 5, 22, 0	0, 0, 0, 0, 0 0, 0, 1
Exp. 3	ND	4, 7, 2, 7	ND	0, 2, 0, 0
Exp. 4	0, 0, 0, 0, 1 3, 0, 3, 0, 0 4, 1, 0, 0, 0	ND	0, 20, 2, 10, 8	0, 1, 1, 1, 0
CFU-S per pool (mean $\pm$ s.e.m.)	1.0 $\pm$ 0.3	4.1 $\pm$ 1.1	6.9 $\pm$ 1.8	0.3 $\pm$ 0.1
CFU-S per macro burst	1.0	1.0	0.9	
b Non-irradiated vs irradiated macroscopic burst cells				
	Non-irradiated		Irradiated	
Exp. 5 <i>In vivo</i> irradiation	6, 2, 2, 2, 19, 22, 27		0, 0, 0, 0, 0, 0, 0	
Exp. 6 <i>In vitro</i> irradiation	5, 2, 1, 2, 4		0, 1, 1, 0, 0, 0, 0	

Using an inverted microscope, 9-d-old isolated macroscopic bursts were selected and taken up individually into a sterile, finely drawn out Pasteur pipette containing a small quantity of α -medium. Care was taken to remove each burst in the smallest volume possible (by weighing dishes this was determined to be no more than 1/250th of the total 1.0-ml culture volume) to minimise further any possible contamination with extraneous cells. The required number of macroscopic bursts were placed in a small volume of α -medium on ice and a single cell suspension obtained by gentle aspiration through a no. 21 needle before injection through a no. 26 needle. B6C3F₁ recipient mice were given 850 rad whole-body radiation⁵ followed by up to 0.6 ml of test material injected into the tail vein. For background values aliquots containing 10% of the culture volume (0.1-ml volumes) were picked at random under microscopic visualisation avoiding only macroscopic bursts and other large colonies (>100 cells). This volume represents 3 $\times$ that picked for assays of eight macroscopic bursts and 24 $\times$ that picked for assays of single bursts. S.e.m.s were calculated by pooling data from exps 1–4. *b* In exp. 5, 8 separate pools, each containing 16 macroscopic bursts, were prepared. Each pool was then subdivided equally between two mice. One of these had already been given 850 rad whole-body X-irradiation before injection in the normal fashion. The other was not irradiated until immediately after injection so that in this group both the cells to be assayed and the recipients were exposed to the same 850-rad treatment. In exp. 6, 4 separate pools, each containing 16 macroscopic bursts, were prepared. Half of each pool was divided and assayed for CFU-S in two recipients in the usual fashion. The other half of each pool received 1,000 rad (X ray) *in vitro* before being divided and assayed for CFU-S in two recipients. ND, not determined.

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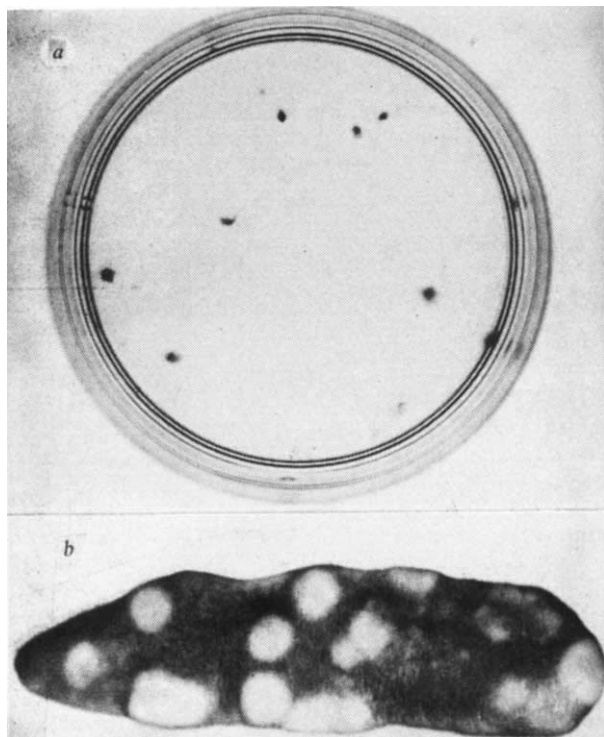


Fig. 2 *a*, Gross appearance of macroscopic bursts from 2-week flask cultured marrow seen in assay dishes after 12 d incubation (2 $\times$). *b*, Gross appearance of spleen (fixed in Tellyesniczky's solution) showing colonies produced 9 d after injection of eight macroscopic bursts (5 $\times$).

repopulating and differentiative potentialities of cells derived from macroscopic burst progenitors.

The broad distribution of CFU-S numbers in individual bursts is of interest, as it approaches that observed for CFU-S self-renewal in spleen colonies^{10,12}. The number of cells plated in burst assay cultures was low. It thus seems most likely that the distribution observed was due to statistical fluctuations¹³ rather than micro-environmental influences¹⁴. This does not negate the possibility that in complex cell systems, stem cell growth and differentiation can be locally influenced by other cell types in the immediate vicinity. It does, however, suggest that their effects can be studied using soluble factors. The high frequency of CFU-S per burst demonstrated here is an order of magnitude higher than that recently reported for mixed haematopoietic colonies of early fetal liver cell origin¹⁵. Thus, experiments to analyse the progeny of individual adult marrow stem cells stimulated *in vitro* are now possible. An immediate application will be to study the possible influence of various molecular factors on the self-renewal and differentiative behaviour of these stem cells.

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Evidence that substance P does not mediate slow synaptic excitation within the myenteric plexus

ELECTRICAL stimulation of presynaptic fibres to the so-called AH¹ or type II² myenteric neurones in guinea pig small intestine evokes a slow excitatory postsynaptic potential (e.p.s.p.) characterised by long-lasting depolarisation associated with increased membrane resistance and augmented excitability³. Two substances have been implicated as possible neurotransmitters for the slow e.p.s.p. Katayama and North reported that application of substance P to myenteric neurones mimicked the slow e.p.s.p.⁴, and J.D.W. and C.J.M. presented several lines of evidence for serotonin as the transmitter substance^{5,6}. We now report that methysergide, a drug which abolishes both the slow e.p.s.p. and the action of exogenous serotonin^{5,6}, does not affect the action of substance P on guinea pig myenteric neurones. The results suggest that substance P is unlikely to be the neurotransmitter which mediates the slow e.p.s.p.

We used conventional methods, which are described in detail elsewhere, to record intracellular electrical activity and to evoke the slow e.p.s.p. in myenteric ganglion cells of guinea pig small intestine³. Substance P from three different sources (Beckman, Serva and Sigma) and methysergide (Sandoz) were applied to the neurones by adding the drugs to the superfusion solution.

We tested substance P 41 times on 35 ganglion cells from 15 guinea pigs. The compound produced a dose-dependent membrane depolarisation in 30 of the ganglion cells and had no effect on 5 of the cells (Figs 1, 2). In 22 neurones, the depolarisation was accompanied by an increase in membrane resistance, with a decrease in 8. The increase in input resistance did not seem to be related to membrane rectification, and probably reflects decreased membrane conductance for potassium, as suggested by Katayama and North⁴. The membrane depolarisation reached a plateau, and after 45–60 s in substance P, repolarisation, apparently reflecting tachyphylaxis, began. The effects of substance P were reversed when the preparations were washed with drug-free Krebs solution (Fig. 1).

Substance P had the same dose-dependent effects in the presence and absence of methysergide (Fig. 2). On all occasions, the membrane depolarisation produced by the three concentrations of substance P in the presence of methysergide was not significantly different ($P > 0.10$) from the values obtained in its

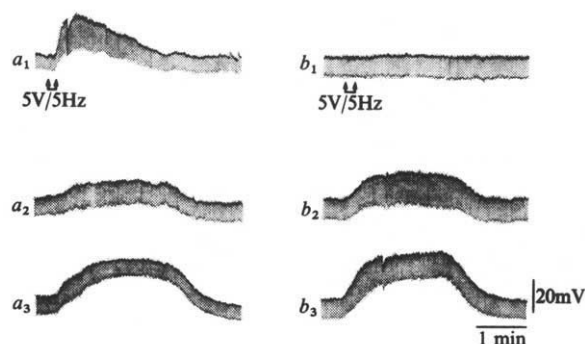


Fig. 1 Effects of methysergide on the slow e.p.s.p. and on the action of substance P in a myenteric neurone of guinea pig small intestine. Each neurone was impaled with the microelectrode and the following procedure carried out. (1) Constant current, hyperpolarising pulses were continuously injected into the neurone at 1-s intervals to monitor changes in the neurone's input resistance. (2) Three concentrations of substance P (10, 100 and 300 nmol l⁻¹) were applied to the neurone in succession, but in variable order. Each concentration of substance P remained in contact with the neurone for 2 min and was then washed from the superfusion system for a period of 5 min with drug-free Krebs solution before addition of the next concentration of substance P. (3) The slow e.p.s.p. was evoked by electrical stimulation of one of the interganglionic fibre tracts that entered the ganglion. (4) Methysergide 20 μ mol l⁻¹ was then added to the superfusion solution. (5) After 5 min in the presence of methysergide, electrical stimulation was again applied to the fibre tract. The same stimulus parameters were used and the stimulating electrode remained in the same position on the fibre tract throughout the experiment on each neurone. (6) The three concentrations of substance P were then applied in the same manner in the continuous presence of methysergide. (7) The methysergide was washed from the superfusion system and electrical stimulation was again applied to the fibre tract. (8) Current-voltage relationship and rectifying properties of the neuronal membrane were examined. The microelectrode was sometimes dislodged from the cell before completion of the above sequence and this is reflected in the numerical data of Fig. 2. We also sometimes repeated the entire sequence on the same cell. *a*₁, Slow e.p.s.p. evoked by application of a short train of stimulus pulses (arrows) to one of the fibre tracts that entered the ganglion. Increased amplitude of the electrotonic potentials produced by current injection (increase in baseline width) reflected an increase in the input resistance of the neurone during the depolarising phase of the e.p.s.p. One spike with a long-lasting hyperpolarising after-potential occurred at the peak of the e.p.s.p. *b*₁, Fibre tract stimulation did not evoke a slow e.p.s.p. in the presence of methysergide. *a*₂ (100 nmol l⁻¹), *a*₃ (300 nmol l⁻¹), Dose-dependent depolarising action of substance P. *b*₂ (100 nmol l⁻¹), *b*₃ (300 nmol l⁻¹). The presence of methysergide did not reduce the dose-dependent action of substance P. This neurone in each case was exposed to substance P for 2 min and then substance P washed from the superfusion system.

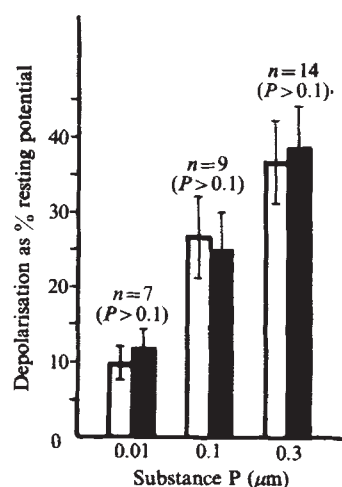


Fig. 2 Dose-dependent effect of substance P on the electrical potential across the membrane of guinea pig myenteric neurones in the presence and absence of 20 μ mol l⁻¹ methysergide. The maximal amount of depolarisation produced by each concentration of substance P is expressed as the per cent of the resting potential before application of the peptide. Results given are mean values, s.e.m.s, number of trials per concentration and the level of statistical significance. The arc sine transformation and Student's *t* statistics⁸ were used to determine the level of significant difference between the means for each concentration of substance P in the presence and absence of methysergide. □, Substance P; ■, Substance P + methysergide.

absence. Similarly, the changes in input resistance produced by substance P were unaffected by methysergide. On the other hand, on the same cells, the presence of methysergide abolished the stimulus-evoked slow e.p.s.p. (Fig. 1), but this effect was reversed by washing the preparation with drug-free solution. Previous studies indicated that this blocking action of methysergide was due to a specific action at serotonin receptors and not a local anaesthetic action, because electrical stimulation still elicited spike discharge in the neurone when the slow e.p.s.p. was blocked in the presence of methysergide^{5,6}.

Our observation that methysergide abolishes the slow e.p.s.p. without affecting the action of substance P makes it unlikely that substance P is the transmitter. Methysergide does block the action of serotonin and this, as well as several other lines of evidence, implicate serotonin as the neurotransmitter⁵. In the brain, the observation that substance P and serotonin occur within the same synaptic vesicles⁷ suggests a functional relationship between the two substances; however, such a relationship has not yet been demonstrated.

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Serum triggers a sequence of rapid ionic conductance changes in quiescent neuroblastoma cells

SERUM is required for the growth of nearly all animal cells in culture, but the mechanisms by which serum interacts with cells are largely unknown^{1,2}. Evidence exists, however, that the primary site of action of the serum constituents is at the plasma membrane. For example, the first detectable events following serum stimulation of resting fibroblasts involve alterations in membrane transport, such as a stimulation of the (Na⁺ + K⁺)ATPase^{3,4} and an increase in the uptake of various nutrients⁵. Most of this evidence has been obtained using tracer flux techniques, but the relatively poor time resolution of this method (of the order of minutes) has precluded detection of dynamic membrane changes that may occur within seconds of serum addition. We have applied intracellular electrophysiological techniques in a search for rapid ionic membrane events following serum stimulation of mouse neuroblastoma cells. These cells stop growing (become 'quiescent') after serum removal and begin to extend neurites, but on re-addition of serum the neurites retract and cell division resumes^{6,7}. Here we report that the immediate consequence of adding fetal calf serum (FCS) to quiescent neuroblastoma cells is a triphasic

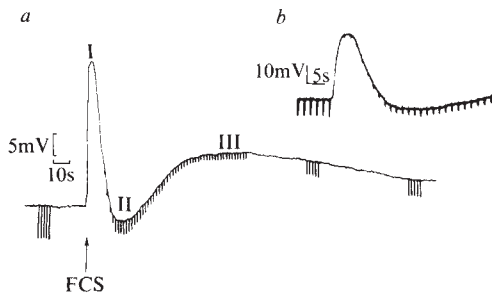


Fig. 1 *a*, Intracellular recording from a N1E-115 cell during addition of FCS (Flow lot no. 2927028) showing the characteristic triphasic voltage response. Arrow indicates time at which the bathing solution was rapidly exchanged for serum-containing solution (final FCS concentration 30%). Membrane resistance was monitored as the voltage response to brief hyperpolarising current pulses (200 ms; 0.1–0.5 nA). Dashed line represents zero potential level. *b*, Oscilloscope recording of the initial phase, clearly showing the changes in membrane resistance. Membrane resistance during peak of phase I was decreased as much as eight-fold (corrected for voltage dependence). Temperature was $36 \pm 1^\circ\text{C}$.

membrane potential response, caused by a series of transient ionic permeability changes, the first of which is a rapid and transient increase in Na^+ conductance. These conductance changes, which are distinctly different from those underlying electrical excitability, seem to be the first events following serum stimulation and may provide a clue to the mode of action of serum growth factors.

Cells of mouse neuroblastoma clone N1E-115 (ref. 8) were used as the model system because their relatively large cell size and their high specific membrane resistance make them most suitable for electrophysiological study^{9,10}. The N1E-115 cells were grown at 37°C on circular glasses (diameter 1.5 cm; plating density 2×10^4 cells per cm^2) for 2 d in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS and 20 mM HEPES (pH 7.4), and were subsequently incubated in serum-free DMEM for at least 24 h. For experimental convenience dimethyl sulphoxide (DMSO, 2% v/v) was sometimes added to the growth medium to induce an enlargement of the average cell size¹¹. DMSO treatment had no effect on the serum-dependent membrane properties.

Intracellular recordings were made with conventional electrophysiological techniques¹⁰, while the cells were bathed either in serum-free DMEM (0.5 ml; $35\text{--}37^\circ\text{C}$) or in a salt solution with the equivalent ionic composition (130 mM NaCl; 5.5 mM KCl; 1.8 mM CaCl_2 ; 0.8 mM MgCl_2 ; 20 mM HEPES, pH 7.4). Heat-inactivated FCS was added by exchanging within a few seconds the serum-free solution for FCS-containing solution.

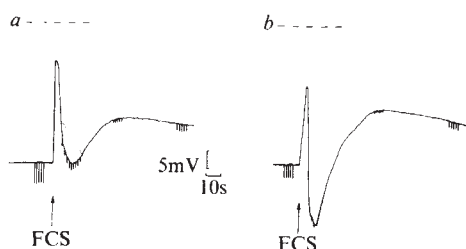


Fig. 2 Effects of increased Ca^{2+} concentration on the serum-induced voltage response. After FCS addition in normal solution (*a*) the culture was washed and exposed to a 10 mM Ca^{2+} saline. The same cell was then re-penetrated and after adjustment of the resting potential to its original level, FCS (containing 10 mM Ca^{2+}) was added again (*b*). Note the marked enhancement of phase II in a hyperpolarising direction, accompanied by a reduction in membrane resistance. Dashed lines represent zero potential level.

N1E-115 cells in serum-free medium maintained resting potentials between -30 and -45 mV and had membrane resistances varying from 10 to $40\text{ M}\Omega$. As illustrated in Fig. 1, addition of FCS (final concentration 30%) immediately elicited a characteristic triphasic voltage response. The initial phase (I) was a rapid depolarisation, reaching maximum values between -5 and -15 mV within a few seconds, accompanied by a substantial fall in membrane resistance. During the next 5–10 s the membrane repolarised to a potential value near the original resting level (phase II), while the membrane resistance only partially recovered. This hyperpolarising phase was followed by a new depolarising phase (III), accompanied by a gradual decrease in membrane resistance. Phase III reached a plateau value of about -25 mV at 40–70 s following FCS stimulation. Finally, both membrane potential and resistance slowly increased again to new steady-state values which were consistently lower than the prestimulation values. This final recovery usually took 4–8 min, but was occasionally much slower (up to 20 min). Peak values of the serum response were dose dependent, showing saturation at a FCS concentration of about 30%. Addition of DMEM containing a high concentration of bovine serum albumin (BSA, final concentration 25 mg ml^{-1} , corresponding to the value in 50% serum) was without any electrical effect. After washing out the FCS-containing medium the serum response could be elicited again in the same cell. This procedure could be repeated several times before the response eventually became 'desensitised'.

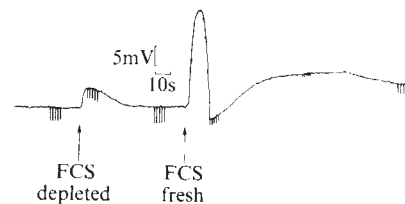


Fig. 3 Effects of growth-depleted and fresh serum (20%) on the same cell. Depleted FCS was obtained by a 3-day exposure of DMEM and 20% FCS to a confluent N1E-115 culture, with daily adjustment of the medium pH to 7.4. Dashed line represents zero potential level.

The ionic mechanisms underlying the sequential phases were evaluated by measuring the null or reversal potential of the distinct peaks as a function of changes in the external ionic concentrations. The null potential of phase I varied between -5 and $+10$ mV and was dependent on the external Na^+ concentration. A threefold reduction of $[\text{Na}^+]$ (choline substituted) resulted in a 20 mV shift of the null potential. A 10-fold reduction of external $[\text{Ca}^{2+}]$, however, did not significantly influence the properties of phase I. These results indicate that phase I is mainly caused by an increase in Na^+ permeability. This phase is not affected by the Na^+ -channel blocker tetrodotoxin (10^{-6} g ml^{-1}), however, and corresponds to a peak of inward current in voltage-clamp conditions (not illustrated). Thus, it follows that the serum-activated Na^+ conductance is clearly different from the voltage-dependent Na^+ channel underlying the generation of the neuroblastoma action potential¹⁰.

Phase II reached maximum hyperpolarising values of up to -45 mV. In view of the reduced resistance value this suggests an increase in permeability to K^+ , which is the only ion with an equilibrium potential (E_K) more negative than the resting potential ($E_K = -75$ mV (ref. 10)). The activation of one component of the K^+ permeability in neuroblastoma cells has been found to depend on Ca^{2+} influx¹². Enhancing the driving force for Ca^{2+} influx by increasing external $[\text{Ca}^{2+}]$ from 1.8 to 10 mM resulted in a striking shift of the peak value in a hyperpolarising direction towards E_K , accompanied by a further

decrease of the membrane resistance (Fig. 2). Maximum peak II values in high $[Ca^{2+}]$ solution approached -60 mV and were reduced by about 40 mV following a 10-fold increase in external $[K^+]$.

The dependence of phase II on both external Ca^{2+} and K^+ strongly suggests that this phase reflects an increase in K^+ permeability, accompanied or preceded by a net Ca^{2+} influx into the cytoplasm. The resulting increase in intracellular $[Ca^{2+}]$ is then thought to underly the Ca^{2+} -dependent membrane hyperpolarisation¹².

The reversal potential of phase III was around -25 mV and was sensitive to changes in external $[Na^+]$ and $[K^+]$, whereas changes in external $[Ca^{2+}]$ were without significant effect. Quantitative evaluation of the ionic selectivity during phase III is difficult, however, as the internal ionic concentrations are likely to change during this prolonged phase of ionic 'leakiness'. We can state, nevertheless, that both Na^+ and K^+ permeability are enhanced during phase III.

The present results are the first direct evidence that serum rapidly triggers dynamic membrane permeability changes to Na^+ , Ca^{2+} and K^+ in a specific temporal sequence. In the only previously published electrophysiological study on growth stimulation, serum has been reported to depolarise the membrane of embryonic rat cells, but the underlying ionic mechanisms have not been elucidated¹³. Interestingly, the present data are reminiscent of the first detectable events occurring at egg fertilisation. Sperm-egg interaction triggers a transient 'fertilisation potential', brought about largely by a rapid increase in Na^+ permeability¹⁴.

A major and intriguing question now is to what extent the rapid serum effects are involved in the triggering of subsequent biochemical events, in particular those which ultimately lead to the initiation of DNA synthesis and cell division. For example, the rapid Na^+ conductance changes might lead to a stimulation of Na^+ -coupled transport processes and to an increase in $(Na^+ + K^+)ATPase$ activity¹⁵. Figure 3 shows that application of growth-depleted FCS, incapable of supporting growth, fails to induce a significant electrophysiological response. This result supports the view that the rapid ionic events are due to the growth-promoting factors in serum. Future investigations of early changes in membrane-linked events as well as characterisation of the serum factors involved should clarify the possible role that the phenomena described here have in growth control.

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Gramicidin A crystals contain two cation binding sites per channel

GRAMICIDIN A, a pentadecapeptide antibiotic from *Bacillus brevis*, dimerises to form transmembrane channels in biological and synthetic membranes¹⁻⁴. These channels are permeable to alkali cations and protons, impermeable to anions and blocked by calcium ions⁵⁻⁸. The high transport rate ($\sim 10^7$ ions s^{-1}), moderate selectivity and simplicity of the gramicidin channel make it an attractive model for elucidating the mechanism of ion transport in biological membranes. Our previous X-ray crystallographic study showed that crystals of native gramicidin and of its complex with caesium ion contain cylindrical channels which are formed from helical dimers of gramicidin⁹. We also found that the binding of caesium leads to a large conformational change: the length of the channel decreases from 32 to 26 Å, whereas the diameter of the cylinder formed by the peptide main chain atoms increases from about 5 to 6.8 Å. We report here X-ray studies showing that the same conformational change is induced by the binding of potassium ion. Furthermore, the isomorphism of the K^+ -complex and Cs^+ -complex crystals enabled us to calculate difference Fourier projections and define the location of the ion-binding sites.

Crystals of the K^+ -complex of gramicidin were grown at room temperature over a 1-yr period from a solution of 21 mM KSCN and 21 mM gramicidin (commercial mixture of gramicidins A, B and C from ICN Life Sciences Group) in methanol. X-ray diffraction data to 2.5 Å resolution were collected as described previously⁹. The unit cell dimensions of the K^+ -gramicidin crystal are virtually the same as those of Cs^+ -gramicidin (Table 1). Many intensity changes due to the substitution of K^+ for Cs^+ , a difference of 36 electrons, are evident in the $0kl$ precession photograph shown in Fig. 1. A three-dimensional difference Patterson map was calculated using $(|F_{Cs}| - |F_K|)^2$ as coefficients. The Harker peaks showed that the space group is $P2_12_12_1$, resolving an earlier ambiguity⁹. The 12 highest peaks in the difference map at 5 Å resolution were interpreted in terms of Harker peaks and cross peaks arising from four cation-binding sites in the asymmetric unit. The positions of anions could not be determined because the thiocyanate counterion was the same in both crystals. The cation-binding sites were refined individually, then in pairs, and finally all together at 5 Å and at 3 Å resolution, using the three centrosymmetric projections by the method of Dickerson *et al.*^{10,11}. When the occupancies and temperature factors of the four sites were constrained to be equal, the centric R factor¹² was 0.36 at 3.0 Å resolution, and the Kraut R factor¹³ was 0.088. The centric R factor dropped to 0.34 when the individual occupancies and temperature factors were allowed to vary. Table 2 gives the coordinates of these sites. Phases of the

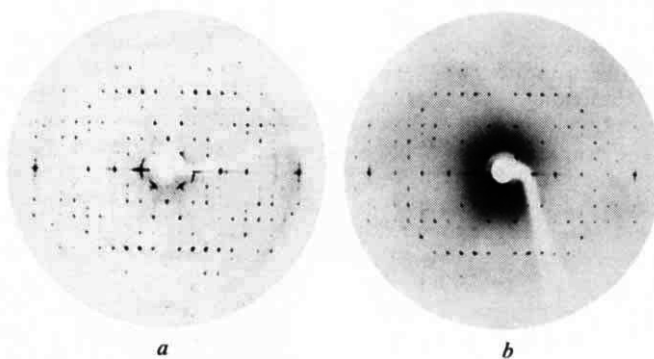


Fig. 1 $0kl$ Precession photographs of crystals of K^+ -gramicidin (a) and Cs^+ -gramicidin (b). The average difference in structure factor between these isomorphous crystals is 16%. A number of significant intensity changes are evident by visual comparison of the two patterns.

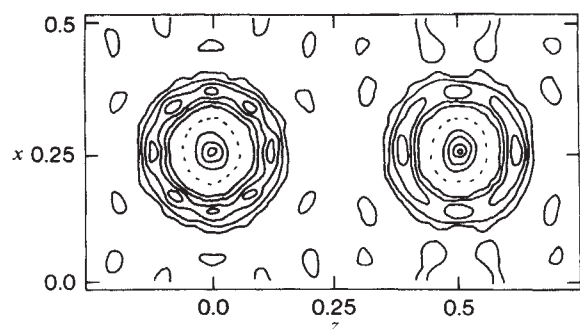


Fig. 2 Centric y-axis projection Fourier map of K^+ -gramicidin at 2.9 Å resolution.

centric reflections were then calculated by the single isomorphous replacement method. Projection difference Fourier maps showed no additional sites. There remains an ambiguity in the phase of each general reflection when only a single isomorphous heavy atom derivative is available. As the heavy atom array is nearly centrosymmetric, a three-dimensional Fourier map calculated from this single derivative is also nearly centric and hence not interpretable at the atomic level.

A projection Fourier map of the K^+ complex is shown in Fig. 2. The asymmetric unit contains two cylindrical channels. Each channel consists of two polypeptide chains and contains two bound cations. The central density in each channel corresponds to the bound ions. The channels at $z = 0$ and $z = 0.5$ are not crystallographically equivalent but appear very similar in projection (Fig. 2). They give rise to a pseudo-cubic-centred and pseudo-tetragonal packing⁹, and so their structures must be nearly the same. The axes of the channels are coincident with crystallographic 2_1 axes, thus limiting the maximum length of a channel to 26 Å, half of the 52 Å unit cell length. The pair of ion sites at $z = 0$ and $z = 0.5$ are related by a translation of nearly 0.25 in y , supporting the notion that the crystallographically independent channels have very similar structures.

Table 1 Unit cell parameters of crystals of K^+ - and Cs^+ -gramicidin

Ion	Space group	<i>a</i>	<i>b</i> *	<i>c</i>
K^+	$P2_12_12$	32.11 Å	52.23	31.09
Cs^+	$P2_12_12$	32.07 Å	52.29	31.20

* Channel axis.

Conductance and spectroscopic studies of analogues of gramicidin A indicate that the two strands in the dimeric channel are antiparallel¹⁴⁻¹⁶. Hence, the two cation-binding sites in a channel are probably equivalent. The two possible symmetric arrangements of these sites are depicted in Fig. 3, which shows that they are either 2.5 Å from the ends of the channel or, alternatively, 2.5 Å on each side of the centre of the channel. Our finding of two cation-binding sites per channel in the crystalline state agrees with $^{86}Rb^+$ flux studies¹⁷ showing the bi-directional fluxes in the gramicidin channel in bilayer membranes are not independent and that two sites are simultaneously occupied. Moreover, the stoichiometry of K^+ binding to gramicidin in methylene chloride is one per polypeptide chain¹⁸, and so two sites per dimeric channel are expected. The dependence of the conductance on the cation concentration also shows that at least two cations can simultaneously interact with the gramicidin channel^{19,20}.

The binding of K^+ , like that of Cs^+ , shortens the channel from 32 to 26 Å and widens its diameter from 5 to 6.8 Å. The diameter cited here refers to the approximate average distance between the centres of the peptide backbone atoms on either side of the channel. The space available for ion binding is about 3.0 Å smaller (the sum of the van der Waals radii of the atoms on either side of the channel). It is interesting that the conformational changes elicited by the binding of these ions are

Table 2 Cation-binding sites in an asymmetric unit in crystals of K^+ - and Cs^+ -gramicidin

Site	<i>x</i>	<i>y</i> *	<i>z</i>
1	8.1 Å	-1.1 Å	-0.2 Å
2	8.0	4.2	0.3
3	7.9	11.6	16.5
4	8.5	16.9	16.1

The unit cell contains a total of 16 cation-binding sites. Two of the four sites in an asymmetric unit are situated near ($x = 0.25$; $z = 0$) and separated by 5 Å along the channel axis y , and another two sites are located near ($x = 0.25$; $z = 0.5$) and separated by 5 Å in y . Each of these four sites gives rise to three additional ones by space group symmetry. In particular, each site is repeated by crystallographic symmetry at the same x and z coordinates and translated by $y = 0.5$ (26 Å). Hence, each site is separated from its neighbours along the y -axis by 5 Å on one side and by 21 Å on the other side.

virtually identical although their ionic radii differ significantly (1.33 Å for K^+ , 1.67 Å for Cs^+ , ref. 21). We have also found that a new tetragonal form of a $CsSCN$ complex of gramicidin is isomorphous with a thallous acetate complex.

Our results thus far are consistent with the existence of two major conformations for the gramicidin channel, an ion-free conformation observed in two crystal forms⁹, and an ion-bound conformation observed in the crystal complexes with Cs^+ , K^+ and Tl^+ . The ion-free conformation seems to be substantially independent of whether the solvent is methanol, ethanol or water. The helical Patterson peaks are the same for the methanol and ethanol crystals⁹. Furthermore, ethanol can be exchanged for 50% ethanol-50% H_2O with almost no change in the diffraction pattern. The large structural difference between forms with and without ions is likely to be due to a rearrangement in the hydrogen-bonding pattern, similar to that observed in valinomycin, an ion-carrier^{22,23}. Some of the peptide $C=O \cdots H-N$ hydrogen bonds are probably broken as the peptide carbonyl oxygens tilt inwards to coordinate the incoming cation. These interactions could not occur in the 5 Å diameter of the native channel because of steric hindrance. It seems likely that the gramicidin channel widens on binding

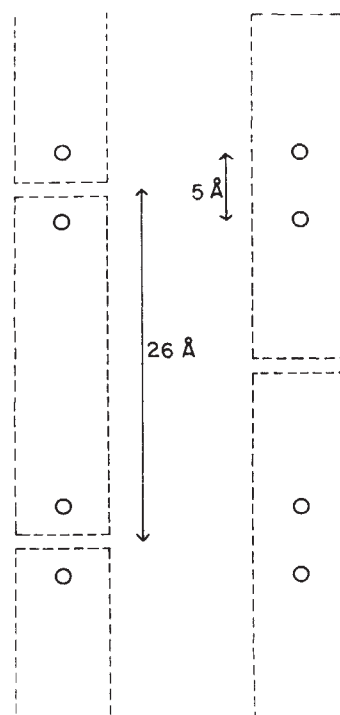


Fig. 3 Schematic drawing showing the two possible symmetric arrangements of the cation-binding sites in a gramicidin channel. Adjacent sites are separated by 5 Å and by 21 Å in a 26 Å-long channel.

cations to permit peptide carbonyl-metal coordination, which compensates for some of the free energy lost in partially dehydrating the transported ion.

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Seminalplasmin—an antimicrobial protein from bovine seminal plasma which acts in *E. coli* by specific inhibition of rRNA synthesis

SEMINAL PLASMA—the fluid in which spermatozoa are ejaculated—has been known to inhibit reversibly both RNA and protein synthesis in sperm cells¹⁻³. Here we report the isolation from seminal plasma, purification to homogeneity, and characterisation of a protein which we have called 'seminalplasmin', that specifically inhibits rRNA synthesis in *Escherichia coli* and is highly antimicrobial.

As transcription and translation in bovine spermatozoa are exclusively mitochondrial^{4,5} and as there are close similarities between mitochondrial and prokaryotic transcription and translation, we used inhibition of the growth of *E. coli* to assay seminalplasmin during purification. Dialysed bovine seminal plasma was passed through a DEAE-Sephadex column and seminalplasmin was purified to homogeneity from the unadsorbed fraction by successive fractionation on a CM-Sephadex, a Sephadex G-75 and a [5'-(p-aminophenylphosphoryl)uridine-2'(3')-phosphate]-agarose (APU-agarose) affinity column (see Fig. 1). The use of the latter column removed traces of RNase SPL⁶. The DEAE-Sephadex column removed an inhibitor, 'antiseminalplasmin', which we have partially purified. Seminalplasmin obtained after the affinity chromatography was

Table 1 Effect of seminalplasmin at various stages of purification, on the growth of *E. coli*

Stage of purification	Concentration of protein ($\mu\text{g ml}^{-1}$)	Inhibition of the growth of <i>E. coli</i> (%)
CM-Sephadex peak 4	40	100
	20	100
	10	40
	5	0
Sephadex G-75 fraction C	40	100
	20	100
	10	90
	5	50
Homogeneous seminalplasmin (after affinity chromatography)	40	100
	20	100
	10	90
	5	50

Aliquots of a logarithmically growing culture of *E. coli* W 160-37 (A_{760} , 0.01) were incubated with various concentrations of seminalplasmin-containing protein fraction. After 6 h, the A_{760} of the culture was measured. The absorbance of the culture to which no seminalplasmin was added, A_{760} of 0.5-0.6, was taken as 100 for the purpose of calculation of the percentage inhibition. For details of the fractions used for the growth-inhibition assay, see Fig. 1 and text. Affinity chromatography removed traces of RNase SPL contamination in Sephadex G-75 fraction C which was otherwise pure seminalplasmin. An A_{280} of 1 was taken to be equivalent to 1 mg of protein throughout this study. The composition of the growth medium was as follows (g l^{-1}): NH_4Cl 0.5; $(\text{NH}_4)_2\text{SO}_4$ 0.5; KH_2PO_4 13.6; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.02; $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ 0.0156; glucose 20; L-arginine hydrochloride 0.1. The above assay for growth inhibition would not detect the presence of a small number (<2% of the control) of viable organisms in the culture. Similar results were obtained with the other strains of *E. coli* tried.

homogeneous in both non-SDS and SDS polyacrylamide gel electrophoresis (PAGE)⁷⁻⁹ (Fig. 1), in an analytical ultracentrifuge (sedimentation coefficient, 1.04S), and in an isoelectric focusing run on a column¹⁰ (isoelectric point, 9.8); it was free of any detectable RNase, DNase or protease activity¹¹.

The amino acid composition of seminalplasmin is, in mol per 100 mol of total amino acids (except Trp) was: Lys 12.30; Arg 9.60; His 4.15; Asx 11.26; Glx 4.92; Phe 3.95; Tyr 1.66; Pro 3.13; Cys 2.98; Thr 1.75; Ser 7.20; Gly 5.75; Ala 7.90; Val 0.63; Leu 14.20; Ile 1.80. The protein is rich in basic amino acids and contains no methionine.

The molecular weight of seminalplasmin was 17,000 by SDS-PAGE carried out as in ref. 8; 10,600 by SDS-PAGE carried out according to Laemmli⁹; 8,000 by ultracentrifugation, and 19,800 (minimum value) when calculated from the amino acid composition. The discrepancy between these values may be a reflection of certain uncommon structural features—such as an abnormally high axial ratio—in the protein.

The average yield of pure seminalplasmin was 18 mg per 100 ml of semen (average ejaculate, 4 ml); the total content of seminalplasmin in bovine semen is probably twice as much.

Table 2 Effect of seminalplasmin on the growth of microorganisms

Petri dish no.	Microorganism	Description	Test substance Amount per disk (nmol)	Diameter of the zone of inhibition (mm)
1	<i>Candida albicans</i>	Seminalplasmin	5	15
		Streptomycin	17	0
		Chloramphenicol	53	0
2	<i>Salmonella typhimurium</i>	Seminalplasmin	5	15
		Streptomycin	17	8
		Chloramphenicol	53	15
3	<i>E. coli</i>	Seminalplasmin	5	15
		Streptomycin	17	8
		Chloramphenicol	53	15

Exponentially growing broth cultures were transferred to medium 199-Earle's salts plates (one for each organism) using a heavy inoculum which, on normal growth, would give a 'mat'. Disks impregnated with the stated amount of substance were placed on the surface of the culture just after the inoculation, and the diameter of the clear zone measured after 20 h at 37°C. The strains used were isolated at the Institute of Preventive medicine, Hyderabad, where the testing was done. The two bacterial strains were partially resistant to streptomycin.

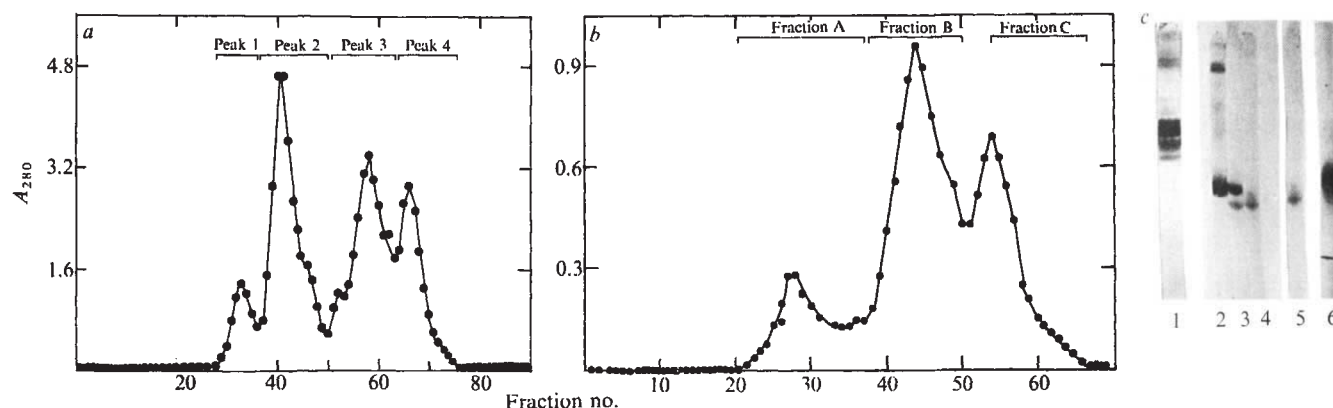


Fig. 1 Purification of seminalplasmin. Seminal plasma (200 ml) obtained from bovine semen following removal of spermatozoa by centrifugation was dialysed against Tris-HCl buffer (0.006 M, pH 7.4) and the dialysed plasma passed through a DEAE-Sephadex column (5×60 cm) equilibrated with the above buffer. The fraction that came out unadsorbed from the DEAE-Sephadex column was chromatographed on a CM-Sephadex C-50 column (2×45 cm) equilibrated with the Tris-HCl buffer and eluted with a linear gradient of 0–0.8 M NaCl in the same buffer. The peaks were pooled as marked, dialysed against water and lyophilised to give the crude seminalplasmin. In some runs, peaks 3 and 4 were not separated. Peaks 1 and 2 had no detectable seminalplasmin activity, while peak 3 had some activity which was possibly due to cross contamination with peak 4. The crude seminalplasmin (peak 4 of a, 100 mg; where peaks 3 and 4 were not separated, the trailing part of the combined peak was used) was then chromatographed on Sephadex G-75 (2.6×170 cm) equilibrated with Tris-HCl buffer (pH 7.4) and eluted with the same buffer. The pooled fractions A, B and C were dialysed separately against water and lyophilised. Fraction C was dissolved in 0.02 M acetate buffer (pH 5) and chromatographed on a APU-agarose affinity column (0.8×6 cm) equilibrated with the acetate buffer. The column was washed with the same buffer; the unadsorbed fraction consisted of pure seminalplasmin (the adsorbed fraction contained RNase SPL). a and b, typical CM-Sephadex and Sephadex G-75 column runs, respectively. c, Polyacrylamide gel electrophoresis runs of various fractions. (1)–(5), Non-SDS PAGE⁷; (6) SDS-PAGE⁸. (1) CM-Sephadex peak 4; (2) Sephadex fraction A; (3) Sephadex fraction B; (4) Sephadex fraction C; (5) and (6) the part of Sephadex fraction C that was unadsorbed on the APU-agarose affinity column (pure seminalplasmin). (1) Expt 1; (2) to (5) expt 2; (6) expt 3. Each gel was loaded with 100 μ g of protein. The gels were stained with Coomassie blue (0.1%) in ethanol:water:acetic acid (45:45:10 v/v). Several protein fractions which could be separated on non-SDS PAGE in (1) moved together on SDS-PAGE done as in (6).

Seminalplasmin potently inhibited the growth of Gram-positive and Gram-negative bacteria and yeasts. In liquid cultures grown in a synthetic medium, starting from an inoculum of 10^6 – 10^7 colony forming units (CFU) ml^{-1} for bacteria and 10^5 – 10^6 CFU ml^{-1} for yeasts, seminalplasmin completely inhibited the growth of *Streptococcus faecalis*, *E. coli* and *Cryptococcus neoformans* at 50–100 μ g (2.5 – 5.0 nmol), 25–50 μ g (1.25 – 2.50 nmol), and 12.5–25 μ g (0.63 – 1.26 nmol) per ml, respectively (see Table 1). Comparable inhibition of growth of organisms considered sensitive to established antimicrobial agents was (nmol ml^{-1}): penicillin and streptomycin, 1.7–8.5; chloramphenicol, 9–27; tetracyclines, 2.2–11; erythromycin, 1.4–2.8; bacitracin, 4.5–9; polymyxin, 0.9–2.2. In disk agar assays, seminalplasmin was active against *Salmonella typhimurium* and *Candida albicans*; it was also active against *Bacillus subtilis* and *Staphylococci* in another type of assay. Seminalplasmin, however, showed no activity against *Pseudomonas* and *Proteus* spp. Results of some typical antimicrobial assays of crude seminalplasmin fractions and the pure protein are shown in Tables 1 and 2.

Seminalplasmin is bacteriocidal and not bacteriostatic. When a culture of *E. coli* W 160-37 containing $\sim 10^7$ cells ml^{-1} was incubated with seminalplasmin (≥ 40 μ g ml^{-1}) for 7 h at 37 °C (when the control contained 2.3×10^8 cells ml^{-1}), and then 50 μ l of the culture plated on nutrient agar, complete inhibition of growth was normally obtained. Occasional colonies of apparently seminalplasmin-resistant cells were seen.

The concentration of seminalplasmin needed to inhibit the growth of *E. coli*, as assayed in Table 1, depended on the number of cells present in the culture; whereas for 10^7 cells ml^{-1} , the concentration required was 10–20 μ g ml^{-1} , for 5×10^7 cells ml^{-1} , 40–50 μ g ml^{-1} , for 10^8 cells ml^{-1} , 60–70 μ g ml^{-1} , and for 2×10^8 cells ml^{-1} , 100–110 μ g ml^{-1} of seminalplasmin was required.

Seminalplasmin is relatively heat stable; when *E. coli* cells (10^7 ml^{-1}) were grown in the presence of seminalplasmin (40 μ g ml^{-1}) heated at 90 °C for 10 min, over 95% inhibition of growth was obtained at 6 h, compared with 100% inhibition with untreated seminalplasmin. Lyophilised, pure seminalplasmin is stable for at least 2 yr at 4 °C; in aqueous solution it is stable for at least 6 months when stored frozen.

A 52% inhibition of the synthesis^{3,4} of RNA in bovine spermatozoa, measured over a period of 2.5 h, was seen with seminalplasmin (1 mg ml^{-1}) preincubated with the cells for

30 min, suggesting that at least part of the total transcription-inhibiting activity of bovine seminal plasma is due to seminalplasmin. Seminalplasmin had no effect on respiration in spermatozoa studied over a period of 2 h using fructose (the semen sugar) as the oxidisable substrate.

Seminalplasmin (100 μ g ml^{-1}) had no effect on the rates of synthesis of DNA and protein in *E. coli* W160-37—at least up to 15 min—in pulse-labelling experiments (Fig. 2a, c). The rate of incorporation of ^3H -uridine into RNA, however, progressively fell to about 50% of the control at 8 min (Fig. 2b); beyond this

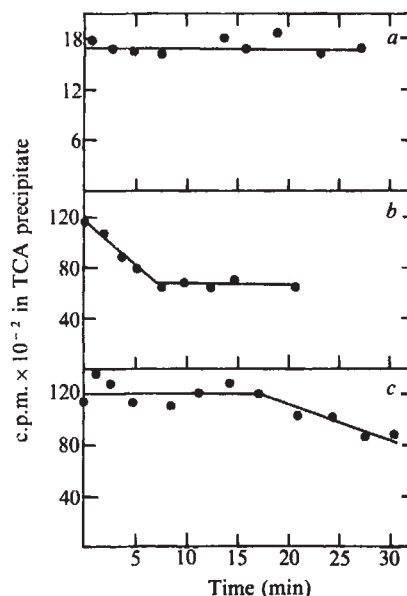


Fig. 2 Effect of seminalplasmin on the rate of synthesis of DNA (a) RNA (b) and protein (c). a, A sample of 6 ml of a logarithmically growing culture of *E. coli* (A_{760} , 0.3) was incubated with seminalplasmin (100 μ g ml^{-1}). At each time point, a 0.5-ml aliquot of the culture was pulse-labelled for 20 s with 13.3 μ Ci (0.41 μ g) of ^3H -thymidine added in 0.2 ml of the growth medium. The reaction was stopped with 5 ml of cold 5% trichloroacetic acid (TCA), and 0.1 ml of 0.5% bovine serum albumin was added. The precipitate was filtered on a Whatman 3 MM filter, washed three times with 10 ml of cold 5% TCA, dried and counted. b, The experimental protocol was as in a but pulse-labelling was with ^3H -uridine (13.3 μ Ci, 0.32 μ g). c, Pulse labelling was as in a but with 1 μ Ci (0.65 μ g) of ^{14}C -leucine. The TCA precipitate was heated with 5% TCA at 95 °C for 30 min and then filtered, washed and counted. Counting efficiency was 40% for ^3H and 80% for ^{14}C .

time no further decrease in rate of RNA synthesis was observed, suggesting that seminalplasmin may be specifically inhibiting the synthesis of a certain type(s) of RNA. In pulse-labelling experiments such as those shown in Fig. 2b, the label is known to be roughly equally distributed between mRNA and rRNA. Since protein synthesis was not affected for at least 20 min, it seemed unlikely that seminalplasmin was inhibiting the synthesis of mRNA. In *E. coli* cells (A_{760} , 0.2) preincubated with seminalplasmin ($170 \mu\text{g ml}^{-1}$) for 15 min, the induction of β -galactosidase with isopropylthiogalactoside was also normal. We showed that seminalplasmin specifically inhibits the synthesis of rRNA in *E. coli*, by analysing on PAGE the RNA labelled in 20 min in the presence of seminalplasmin ($100 \mu\text{g ml}^{-1}$) in a growing culture of *E. coli* which had been preincubated with the protein for 15 min (Fig. 3). Seminalplasmin inhibited the labelling of rRNA by 85%; tRNA synthesis was inhibited by $\sim 10\%$, probably through inhibition of the transcription of genes for tRNA that are a part of the rRNA cistrons in *E. coli*¹². In line with these observations, addition of seminalplasmin ($100 \mu\text{g ml}^{-1}$) to *E. coli* cultured in the presence of ^3H -uridine almost completely stopped the increase in the label appearing in total RNA after 12 min in cells growing in the absence of chloramphenicol (Fig. 4a), and completely stopped it in chloramphenicol-treated non-growing cells (Fig. 4b) in which the RNA that is labelled is almost entirely transcribed from rRNA cistrons. In cells treated with chloramphenicol and seminalplasmin, the RNA labelled in the first 12 min (the time presumably required for building up of the intracellular concentration of seminalplasmin necessary for its full inhibitory effect) was subsequently degraded (Fig. 4b); this degradation—also observed in the presence of rifampicin instead of seminalplasmin¹³—may be related to the instability of rRNA synthesised in the presence of chloramphenicol, hence not protected by ribosomal proteins.

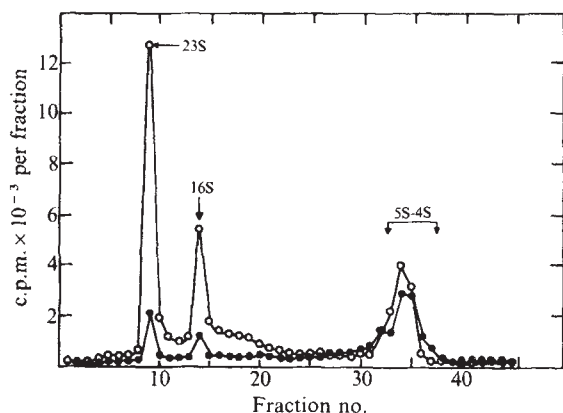


Fig. 3 Fractionation by polyacrylamide gel electrophoresis of *E. coli* RNA synthesised in the presence or in the absence of seminalplasmin. Aliquots of 5 ml of a logarithmically growing culture of *E. coli* (A_{760} , 0.30) were incubated with or without seminalplasmin ($200 \mu\text{g ml}^{-1}$). At 10 min, ^3H -uridine ($45 \mu\text{Ci}$, $10.1 \mu\text{g}$) was added to both the aliquots. At 35 min, $50 \mu\text{l}$ of 2% sodium azide and 1 mg of ^{12}C -uridine were added and the solution was immediately kept in an ice-salt bath. Unlabelled *E. coli* RNA (2 mg) was then added and RNA isolated from both the samples by extraction with aqueous phenol at pH 5.2. RNA solutions were dialysed and fractionated on a 2.5% polyacrylamide gel, and the gels cut and counted. Arrows indicate the positions of the carrier (non-radioactive) 23S, 16S and SS-4S RNA fractions visualised by staining with methylene blue (0.01% in 0.01 M acetate buffer, pH 4.7). $\circ$, Without seminalplasmin; $\bullet$, with seminalplasmin.

In *E. coli* in the presence of a high concentration of seminalplasmin ($200 \mu\text{g ml}^{-1}$), the rate of protein synthesis also started to decrease after 10 min; the fall was linear, reaching 50% of control at 26 min. In the presence of $100 \mu\text{g}$ of seminalplasmin per ml, the rate of protein synthesis started to fall (linearly) only after 15–20 min, reaching 50% of the control after 40 min. An eventual decline in the rate of protein synthesis is to be expected as a consequence of cessation of rRNA synthesis.

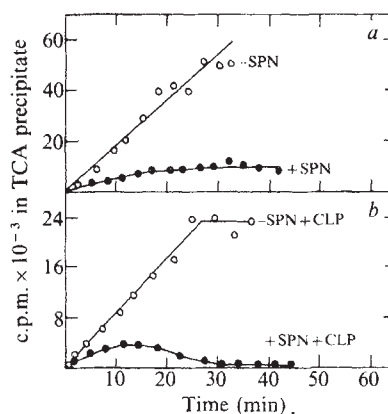


Fig. 4 a, Effect of seminalplasmin on RNA synthesis in *E. coli*. Aliquots of 11 ml of a logarithmically growing culture of *E. coli* (A_{760} , 0.3) were incubated for various periods at 37°C with (or without) seminalplasmin ($100 \mu\text{g ml}^{-1}$), in the presence of ^3H -uridine ($3.9 \mu\text{Ci ml}^{-1}$, $7.88 \mu\text{g ml}^{-1}$). At each time point, a 0.5-ml aliquot from each of the incubated cultures was precipitated with 5 ml of ice-cold 5% TCA containing an excess of ^{12}C -uridine; the radioactivity (x) of the precipitate was taken as a measure of labelling of the total nucleic acid. Another 0.5-ml aliquot from the culture was treated with an equal volume of 1 M KOH for 18 h at 37°C , neutralised and then made 5% with respect to TCA; the radioactivity (y) in the precipitate gave the extent of labelling of DNA. $x - y$ gave the radioactivity in RNA. b, Effect of seminalplasmin on RNA synthesis in a chloramphenicol-inhibited culture of *E. coli*. Aliquots of 11 ml of a logarithmically growing culture of *E. coli* (A_{760} , 0.26) were incubated at 37°C with (or without) seminalplasmin ($212 \mu\text{g ml}^{-1}$), in the presence of chloramphenicol ($149 \mu\text{g ml}^{-1}$) and ^3H -uridine ($1.86 \mu\text{Ci ml}^{-1}$, $7.48 \mu\text{g ml}^{-1}$). Aliquots were taken from each culture at various times and the radioactivity in RNA and DNA determined as in (a). SPN, seminalplasmin; CLP, chloramphenicol.

The antimicrobial and *in vivo* rRNA-transcription inhibiting activities of seminalplasmin were completely abolished by pronase, trypsin, chymotrypsin or papain.

Figure 5 shows the effects of adding pronase ($30 \mu\text{g ml}^{-1}$) to cultures of *E. coli* (A_{760} 0.01) exposed to seminalplasmin ($50 \mu\text{g ml}^{-1}$) for various intervals between 0 and 60 min. Addition of pronase at 0 min showed no inhibition of growth. Increased inhibition of growth was obtained as the time of exposure to seminalplasmin in the absence of pronase increased. A 30-min exposure to seminalplasmin ($50 \mu\text{g ml}^{-1}$) seemed to be sufficient to allow the *E. coli* cells at $\sim 10^7$ cells ml^{-1} to take up enough seminalplasmin to inhibit the growth completely. The experiments described in Figs 2 and 4 suggest that, at a higher concentration ($100 \mu\text{g ml}^{-1}$), enough seminalplasmin enters the cells ($\sim 3 \times 10^8 \text{ ml}^{-1}$) in 8–12 min to exert its maximal inhibitory effect on rRNA synthesis.

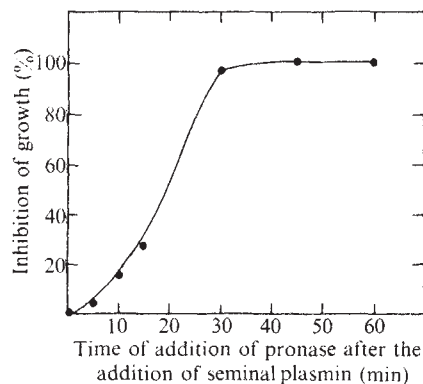


Fig. 5 Effect of pronase on the growth of *E. coli* preincubated with seminalplasmin for various periods. To a set of tubes (one for each time point) containing the growth medium (5 ml) and seminalplasmin ($50 \mu\text{g ml}^{-1}$), was added 1 ml of a logarithmically growing culture of *E. coli* (A_{760} , 0.06). The tubes were incubated at 37°C and, at each time point, pronase ($30 \mu\text{g ml}^{-1}$) was added to one tube. After 6 h, A_{760} of all the cultures was measured. For calculation of the percentage inhibition of growth in the seminalplasmin and pronase-treated cultures, the growth (A_{760} , 0.5–0.6) obtained in a control culture to which no seminalplasmin but pronase was added, was taken as 100. Pronase, at the concentrations used, had no effect on the growth of *E. coli*; at higher concentrations ($100 \mu\text{g ml}^{-1}$) it stimulated growth.

Seminalplasmin is apparently the fourth antimicrobial protein to be isolated from mammalian sources—the others being lysozyme, β -lysin, and a recently described protein from polymorphonuclear leukocytes¹⁴. After colicin E₃, it appears to be the first protein for which there is strong evidence that it enters an intact bacterial cell. As seminalplasmin acts on a variety of microorganisms, it is unlikely that its entry into the cell—unlike that of colicin E₃—is receptor-mediated. Seminalplasmin is also the first inhibitor shown to specifically inhibit the transcription of rRNA in a whole cell.

We first considered the possibility that seminalplasmin may have a role in fertilisation. In a year-long experiment we looked for a correlation between the net antibacterial activity (a function of the ratio of seminalplasmin to antiseminalplasmin, and the amount of each, in seminal plasma) of over 1,000 samples of bovine seminalplasma derived from 126 animals belonging to eight different breeds of two different bovine species (buffalo-bull and cow-bull), and the fertility of semen samples from which the seminal plasma was obtained (P.M.B. *et al.*, unpublished). The fertility was determined by artificial insemination of nearly 2,200 cows and buffaloes. To allow for seasonal variations, the samples were collected over the entire calendar year. Computer analysis of the data has shown no simple correlation between the net antibacterial activity of seminal plasma and the fertilising ability of the semen sample from which the particular batch of seminal plasma was derived.

It is tempting to speculate that the function of seminalplasmin is to act as an antimicrobial agent. It could provide protection either to the male or to the female through deposition of seminal plasma in the reproductive tract during sexual intercourse, or to both. If the natural function of seminalplasmin were to provide protection to the female, one might expect to find a higher incidence of microbial infection of the female genital tract in women who are celibate or have sexual intercourse only rarely, than in women who have frequent sexual intercourse. We are investigating this possibility.

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Seminalplasmin is a potent inhibitor of *E. coli* RNA polymerase *in vitro*

SEMINALPLASMIN, a protein isolated from bovine seminal plasma, specifically and almost completely inhibits synthesis of rRNA in whole *Escherichia coli* cells at extracellular concentrations lower than those needed for bacteriocidal action of many established antibacterial agents¹. We show here that seminalplasmin also strongly inhibits the transcription of various natural and synthetic templates by *E. coli* RNA polymerase *in vitro*, and that it does so by binding strongly to the polymerase. Seminalplasmin is, to our knowledge, the first protein isolated from an eukaryote to inhibit RNA polymerase. There is, however, a protein synthesised in *E. coli* following a phage infection and coded by the phage genome, which appears to inhibit *E. coli* RNA polymerase².

Pure seminalplasmin was prepared as described in the accompanying paper¹. Figure 1 shows the effect of seminalplasmin on the transcription of various templates by *E. coli* RNA polymerase (holoenzyme), following a 12–30-min incubation of seminalplasmin with the polymerase alone (Fig. 1A–H) or with the template (Fig. 1G, H). With a polymerase concentration of 25 $\mu\text{g ml}^{-1}$ (in some cases, 50 $\mu\text{g ml}^{-1}$), the concentration of seminalplasmin required, in $\mu\text{g ml}^{-1}$, for 50% inhibition of the transcription of the various templates, following incubation of seminalplasmin with the polymerase for 30 min (15 min for poly(dI-dC)), was: poly(dA) 50; poly(dT) 50; poly(dA-dT) 50; poly(dG-dC) 100; poly(dI-dC) 100; poly(dA)·poly(dT) 30; T7 DNA 25; and calf thymus (CT) DNA 25. With all the templates except poly(dA-dT), poly(dG-dC) and poly(dI-dC), over 90% inhibition of transcription was obtained with seminalplasmin at 100 $\mu\text{g per ml}$ (5 nmol per ml, assuming a MW of 19,800; see ref. 1). For poly(dA-dT), poly(dG-dC) and poly(dI-dC), maximum inhibition obtained was 50–60% at a seminalplasmin concentration of 100 $\mu\text{g ml}^{-1}$. Heating seminalplasmin for 10 min at 80 °C destroyed 90% of its transcription-inhibiting activity (Fig. 2).

The following four observations show that seminalplasmin did not possess any RNase, DNase or nonspecific protease activity that could account for its transcription inhibiting activity. (1) Seminalplasmin (130–140 $\mu\text{g ml}^{-1}$) did not release any acid-soluble radioactivity from—or change the polyacrylamide gel electrophoresis (PAGE) pattern of—¹⁴C-poly(rU) (37 μg (80,360 c.p.m.) per ml) or ³H-labelled total denatured *E. coli* RNA (500 μg (360,000 c.p.m.) per ml) in 60 min at 37 °C, in conditions in which RNase A or RNase SPL (ref. 1) (8–12 $\mu\text{g ml}^{-1}$) converted 90–100% of the above substrates to acid-soluble material in 5–10 min. (2) Seminalplasmin (100 $\mu\text{g ml}^{-1}$) released no measurable acid-soluble radioactivity from ³H-poly(dT) (14.8 nmol of TMP residues (1.18 × 10⁶ c.p.m.) per ml) on incubation for 30 min at 37 °C in the transcription buffer described in Fig. 1. (3) Seminalplasmin (50–480 $\mu\text{g ml}^{-1}$) did not affect the enzyme activity of β -galactosidase (5.5 $\mu\text{g ml}^{-1}$; homogeneous on PAGE) or polynucleotide phosphorylase (50 $\mu\text{g ml}^{-1}$), or did not release any 280 nm-absorbing or Folin reagent-positive acid-soluble material from casein (5 mg ml⁻¹) in 20 min at 37 °C. (4) Addition of an excess (200 $\mu\text{g ml}^{-1}$) of bovine serum albumin to the transcription buffer (Fig. 1), did not affect the inhibition by seminalplasmin (100 $\mu\text{g ml}^{-1}$), of the transcription of poly(dT) by RNA polymerase (25 $\mu\text{g ml}^{-1}$).

The following two observations strongly suggest that seminalplasmin inhibits the transcription of various templates by binding to the RNA polymerase. (1) Increase in the concentration of RNA polymerase relieved the inhibitory effect of seminalplasmin on transcription (Fig. 3B). An increase in the concentration of the template had no such effect (Fig. 3C). (2) Incubation of seminalplasmin with the polymerase for 20–

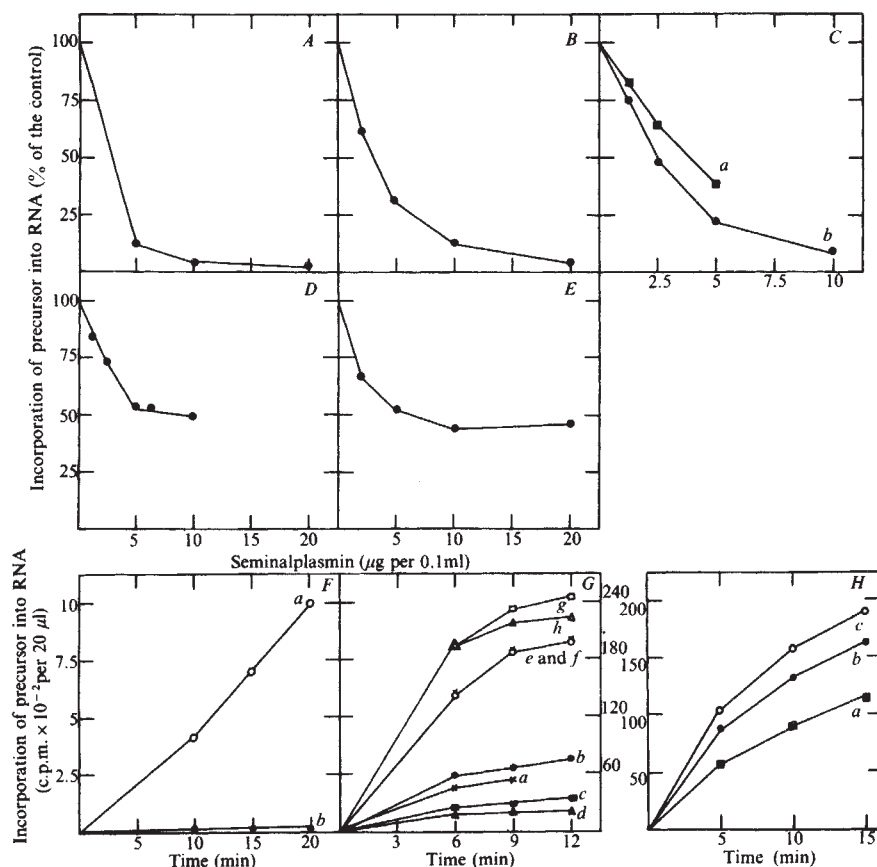


Fig. 1 Effect of seminalplasmin incubated with *E. coli* RNA polymerase, on the transcription of various templates. The reaction mixture contained, in 0.1 ml of Tris-HCl buffer (pH 7.8; for composition, see ref. 3): 0.15 mM of each of the substrates, UTP, GTP, CTP, ATP, and/or ITP, as required (with one of these substrates labelled with ^{14}C or ^{32}P); *E. coli* RNA polymerase (holoenzyme, 5 μg or 2.5 μg); template DNA; and seminalplasmin at the stated concentration. The templates used (in 0.1 ml of the incubation medium) were: T7 DNA (A), 0.244 pmol; CT DNA (B), 2.94 μg ; poly(dA)-poly(dT) (C), 0.074 A_{260} units; poly(dA-dT) (D), 0.1 A_{260} units; poly(dG-dC) (E), 0.1 A_{260} units; poly(dA) (F), 0.2 A_{260} units; poly(dT) (G), 0.5 A_{260} units; poly(dI-dC) (H), 0.05 A_{260} units. In A to E, the polymerase and seminalplasmin were incubated for 30 min, DNA was added, the mixture was incubated for a further 5 min, and the reaction was started by the addition of the substrate mix. In F, the polymerase was incubated for 20 min without (a) or with (b) seminalplasmin (10 μg in 0.1 ml). In G, either the polymerase and seminalplasmin (10 μg in 0.1 ml) were incubated for 12 min (a), or the polymerase, seminalplasmin (10 μg in 0.1 ml) and poly(dT) were incubated for 10 (b), 20 (c) or 30 (d) min; e, f, g and h are controls for a, b, c and d, respectively, in which the polymerase (or the polymerase and the template) was incubated without seminalplasmin. In H, the polymerase and seminalplasmin (10 μg in 0.1 ml) were incubated for 20 min in a; the polymerase and seminalplasmin (10 μg in 0.1 ml) and poly(dI-dC) were incubated for 15 min in b; and the polymerase alone was incubated for 20 min in c which served as the control for a and b. The time of reaction (6 min in A; 10 min in B-E; and the time given on the x-axis in F-H) was counted from the time of the addition of the substrate mix. The substrate mix contained all the four nucleoside triphosphates in the case of A and B; ATP and UTP in C and D; CTP and GTP in E; UTP in F; ATP in G; and ITP and CTP in H. The labelled precursors used were: ^{14}C -UTP (2,808 c.p.m. nmol^{-1}) in A, B, C (graph a) and F; ^{14}C -ATP (2,776 c.p.m. nmol^{-1}) in C (graph b), D and

G; [α - ^{32}P]CTP (24,000 c.p.m. nmol^{-1}) in E and H. The concentration of the polymerase was 2.5 μg in 0.1 ml in A-D; and 5 μg in 0.1 ml in E-H. All incubations were carried out at 37°C. A 20- μl aliquot was withdrawn from the reaction mixture, spotted over a streak of glacial acetic acid on a 10 $\times$ 2 cm strip of Whatman no. 3 filter paper, and chromatographed for 45 min in ethanol: ammonium acetate (1 M) (1:1) solvent. A 1-cm strip was cut along the original spot and the radioactivity in it measured; this radioactivity was taken as the measure of RNA synthesis*. An appropriate control, without seminalplasmin, was run in every case; the radioactivity (in c.p.m.) contained in RNA (20 μl of the incubation mixture) in the control samples was: A 1,500; B 1,256; C(a) 1,468; C(b) 243; D 2,556; E 927. The same batch of RNA polymerase was used in all experiments described here. Its specific activity was 16,000 units per mg assayed with T7 DNA as the template, and it was 95% pure as judged by SDS-polyacrylamide gel electrophoresis; it contained 1 equivalent of the σ subunit. An A_{280} of 1 was taken to be equivalent to 1 mg of seminalplasmin.

30 min before the start of transcription was necessary for optimal inhibition of transcription (Fig. 1). Very much lower inhibition was obtained when seminalplasmin was added at the same time at which the transcription was started (Table 1). The inhibition was virtually abolished if seminalplasmin was added after (Fig. 3D)—or incubated with the template before (Fig. 3A)—the transcription was started.

That seminalplasmin indeed is bound tightly to RNA polymerase was shown by the following experiment. A mixture of the two proteins incubated for 30 min at 37°C (as in Fig. 1) was chromatographed on a column of Sephadex G-75, on which column RNA polymerase and seminalplasmin, when chromatographed separately, were widely separated. RNA polymerase incubated with seminalplasmin (RP-S) was eluted in the

exclusion volume, like RNA polymerase incubated in the absence of seminalplasmin (RP) (Fig. 4). SDS-PAGE runs of the RP-S fractions showed that they contained seminalplasmin (Fig. 5). The subunit pattern, on SDS-PAGE, of RNA polymerase was not altered on binding seminalplasmin, showing that seminalplasmin did not exhibit any specific proteolytic activity towards the polymerase, or remove from it any of its subunits such as the σ factor (Fig. 5). In accord with these observations, incubation of seminalplasmin (50 $\mu\text{g ml}^{-1}$) for 1 h with an excess of RNA polymerase (200 $\mu\text{g ml}^{-1}$, a concentration high enough to overcome the inhibition of transcription by seminalplasmin see Fig. 2B), did not lead to any loss in the transcribing ability of the polymerase.

As expected, the transcribing ability of RP-S, assayed on

Table 1 Effect of seminalplasmin on transcription of various templates by *E. coli* RNA polymerase

Template	Description	Concentration	Substrate	(c.p.m. nmol^{-1})	% Inhibition of transcription by seminalplasmin at:			
					50 $\mu\text{g ml}^{-1}$	100 $\mu\text{g ml}^{-1}$	150 $\mu\text{g ml}^{-1}$	200 $\mu\text{g ml}^{-1}$
T7 DNA	3.7 pmol		^{14}C -ATP	1,640	13	13	25	45
CT DNA	29.4 μg		^{14}C -ATP	1,640	0	16	22	27
Poly(dA-dT)	2 A_{260} units		^{14}C -ATP	1,640	0	0	15	22
Poly(dA)-poly(dT)	2 A_{260} units		^{14}C -ATP	2,776	4	7	—	12
Poly(dA)	2 A_{260} units		^{14}C -UTP	2,833	0	0	0	0
Poly(dT)	4.8 A_{260} units		^{14}C -ATP	2,576	16	22	26	38
Poly(dG-dC)	1 A_{260} unit		[α - ^{32}P]CTP	24,000	0	0	0	0
Poly(dI-dC)	0.5 A_{260} unit		[α - ^{32}P]CTP	24,000	0	0	0	0

The effects of seminalplasmin on transcription were studied as in Fig. 1 in a total reaction volume of 0.1 ml, with the exception that seminalplasmin was added at the same time as the template, and the mixture preincubated only for 5 min before the reaction was started by the addition of substrate. The transcription was continued for 10 min [9 min for T7 DNA and poly(dT)] after the addition of the substrate mix, and 20- μl aliquots were counted for estimation of radioactivity in RNA, as in Fig. 1. The final concentration of RNA polymerase in the incubation mixture was 50 $\mu\text{g ml}^{-1}$.

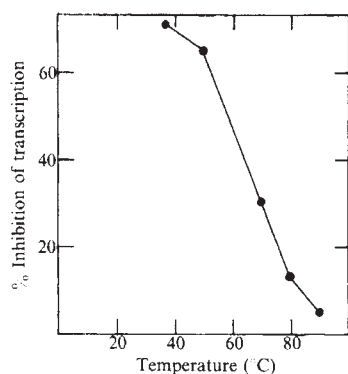


Fig. 2 Effect of heat on the transcription-inhibiting activity of seminal-plasmin. Seminalplasmin (8 μ g), in 75 μ l of Tris-HCl buffer (Fig. 1), was heated for 10 min at the stated temperature and chilled in ice. RNA polymerase (2.5 μ g in 5 μ l of buffer) was added and the mixture incubated for 30 min. T7 DNA (0.244 pmol; 10 μ l) was added, the mixture further incubated for 5 min, and the transcription started by the addition of the substrate mix (10 μ l) and carried out for 9 min, as in Fig. 1. 14 C-UTP was used as the labelled substrate and the inhibition of transcription measured as described in Fig. 1.

poly(dA-dT) (Fig. 4) and poly(dA), was much lower than that of RP. Seminalplasmin apparently binds to *E. coli* RNA polymerase with high affinity: the transcription-inhibitory activity of the RNA polymerase-seminalplasmin complex formed after a 20-min incubation as in Fig. 1, was not affected on incubation of the complex for 20 min in the presence of 1 M NaCl.

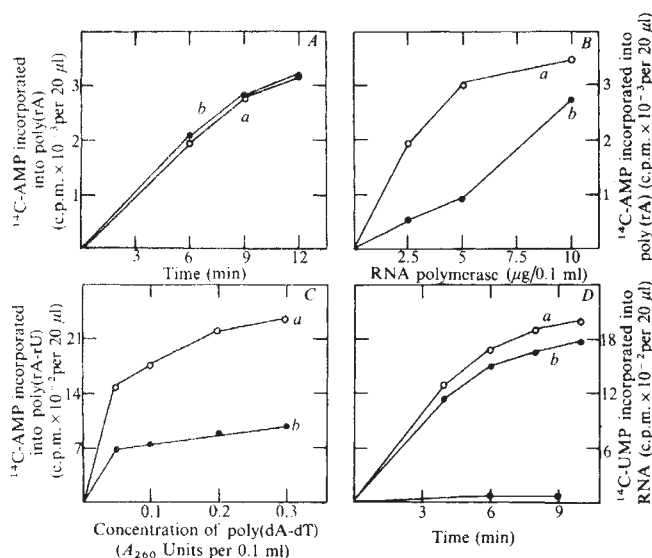


Fig. 3 A, Effect of incubation of the template with seminalplasmin on transcription. Poly(dT) (0.05 A_{260} units) was incubated without (a) or with (b) 10 μ g seminalplasmin for 20 min, RNA polymerase (5 μ g) was then added, the mixture was further incubated for 5 min, and the reaction was started by the addition of 14 C-ATP; 20- μ l aliquots were taken out at various times for measurement of radioactivity in RNA. The other details were as in Fig. 1. B, Effect of increasing the concentration of RNA polymerase on the inhibition of transcription of poly(dT) by seminalplasmin. RNA polymerase was incubated without (a) or with (b) seminalplasmin (5 μ g) for 12 min, poly(dT) (0.025 A_{260} units) was then added, the mixture was further incubated for 5 min, and the reaction was started by the addition of 14 C-ATP and carried out for 9 min. The other details were as in Fig. 1. C, Effect of increasing the concentration of the template on the inhibition of transcription of poly(dA-dT) by seminalplasmin. RNA polymerase (2.5 μ g) was incubated without (a) or with (b) seminalplasmin (5 μ g) for 30 min, the stated amount of poly(dA-dT) was then added, the mixture was incubated for a further 5 min, and the reaction was started by the addition of UTP and 14 C-ATP and carried out for 6 min. Other details were as in Fig. 1. D, Effect of addition of seminalplasmin on the transcription of T7 DNA. The transcription was started as in Fig. 1 but without incubation with seminalplasmin; the reaction mixture contained 2.5 μ g of RNA polymerase. After 2 min, 10 μ g seminalplasmin was added in b (but not in a which served as the control), and the transcription continued. In c, 10 μ g seminalplasmin and 2.5 μ g RNA polymerase were incubated for 30 min. Other details were as in Fig. 1. In all the figures, A-D, the reaction was carried out in 0.1 ml and 20 μ l of the reaction mixture was used for estimation of radioactivity in RNA as described in Fig. 1.

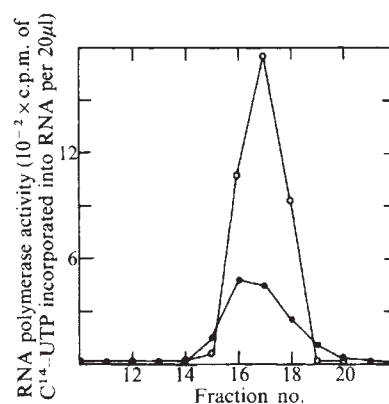


Fig. 4 Chromatography of the RNA polymerase and seminalplasmin complex on Sephadex G-75. RNA polymerase (20 μ g) was incubated without (○) or with (●) seminalplasmin (40 μ g) in Tris-HCl buffer (Fig. 1) in a total volume of 50 μ l, for 30 min at 37°C; 40 μ l of the mixture was then loaded on a Sephadex G-75 column (1 $\times$ 9 cm). The column was eluted with the Tris-HCl buffer and 80 μ l fractions collected. The polymerase activity in each fraction (10- μ l aliquot) was assayed as in Fig. 1, in a total volume of 0.05 ml using poly(dA-dT) as the template; 14 C-UTP (4,000 c.p.m. nmol $^{-1}$) and ATP were used as the substrates, and the reaction was carried out for 10 min after the addition of the substrate mix.

That seminalplasmin inhibits the formation of the first inter-nucleotide linkage (the initiation step in transcription), at least in the case of certain promoters in T7 DNA recognised by the RNA polymerase *in vitro*, was shown by the following experiment done essentially as described in Fig. 1. RNA polymerase (25 μ g ml $^{-1}$) was incubated for 30 min without seminalplasmin (the control), or with seminalplasmin 50 μ g ml $^{-1}$; T7 DNA (1.2 pmol ml $^{-1}$) was then added, the incubation continued for another 10 min, and the transcription started by addition of a mixture of ATP and 14 C-UTP. After allowing transcription for 30 min, a 20- μ l aliquot from each sample was subjected to ascending chromatography on a paper strip (18 $\times$ 2 cm) in water:saturated ammonium sulphate solution:2-propanol (18:80:2 v/v); 1 cm-wide strips were then cut and counted. This system allows separation of pppApU and the triphosphate precursors used³. The presence of seminalplasmin in the incubation mixture caused a 34% reduction in the radioactivity found in the pppApU spot. Seminalplasmin at the concentration used in this experiment inhibited transcription of T7 DNA by 70–85%.

The presence of the template from the start of the incubation mixture caused little change in the inhibitory effect of seminalplasmin on the transcription of templates such as poly(dT), but significantly reduced the inhibitory effect for other templates including poly(dI-dC) (Fig. 1G,H). The reasons for this difference are also not clear.

Seminalplasmin inhibited the transcription of poly(dA-dT) by RNA polymerase core enzyme to the same extent as that by the holoenzyme; no other template was tried with the core enzyme.

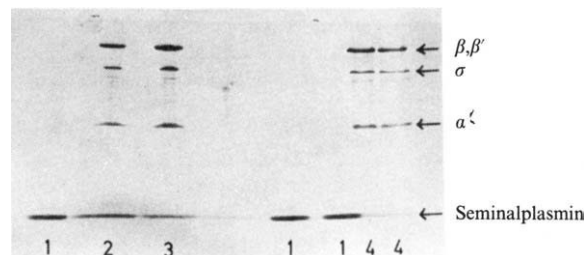


Fig. 5 Fractionation by SDS-PAGE of seminalplasmin-RNA polymerase complex from a Sephadex G-75 column. The complex was formed by incubating seminalplasmin (90 μ g) with RNA polymerase (50 μ g) in 60 μ l of the transcription buffer for 30 min at 37°C, and chromatographed on a Sephadex G-75 column, as in Fig. 4. The polymerase-containing fractions 14–16 and 17–19, eluting in the exclusion volume, were pooled separately, lyophilised, and run on SDS-PAGE⁵ using a 5% (top)–15% (bottom) discontinuous gradient slab-gel. RNA polymerase holoenzyme and seminalplasmin were run on the same gel as controls. 1, Seminalplasmin; 2, fractions 14–16 of Sephadex G-75 column; 3, fractions 17–19 of Sephadex G-75 column; 4, RNA polymerase holoenzyme.

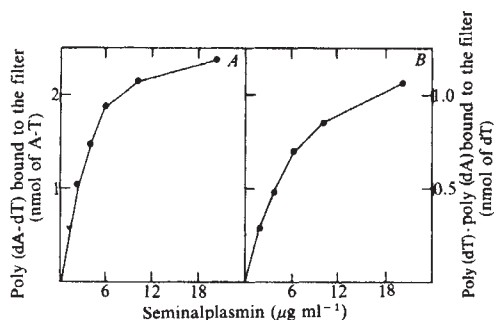


Fig. 6 Binding of seminalplasmin to DNA. ^3H -poly(dA-dT) (2.4 nmol of dA-dT; 8,200 c.p.m.) (A) or ^3H -poly(dT) plus poly(dA) (1.48 nmol of dT; 117,700 c.p.m.) (B) was incubated in 1 ml of Tris-HCl buffer (pH 7.9) containing 0.05 M NaCl and 10 mM MgCl_2 , with the stated amount of seminalplasmin for 30 min at 0°C , and filtered through a Millipore membrane filter ($0.45 \mu\text{m}$). The filter was washed slowly with 5 ml of the ice-cold buffer, dried and counted.

Seminalplasmin also binds strongly to DNA (Fig. 6A, B), a maximum of 1 mol of seminalplasmin being bound per 8 base pairs in the case of poly(dA-dT). (The stoichiometry of interaction of seminalplasmin with poly(dT)·poly(dA) (Fig. 6B) could not be estimated, as the reaction mixture contained free poly(dA) in addition to the complex.) Apparently, this binding is not involved in the inhibition of transcription.

The precise mechanism, through which the binding of seminalplasmin to *E. coli* RNA polymerase leads to loss of enzyme activity remains unclear. It is also not known why seminalplasmin inhibits the transcription of certain templates such as poly(dT), T7 DNA and CT DNA, completely, while only a partial inhibition is observed with DNAs such as poly(dA-dT) containing a repeating dinucleotide sequence.

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Low tryptophan diet decreases brain serotonin and alters response to apomorphine

RATS fed on a low tryptophan diet since weaning have lower levels of brain serotonin than rats maintained on a control diet (18% casein diet)^{1,2}. We have used such rats to investigate the role of the serotonergic system in the regulation of a behaviour primarily controlled by dopaminergic neurotransmission—apomorphine-induced stereotyped behaviour³⁻⁵. This behaviour, which is induced by psychomotor stimulant drugs, including apomorphine and amphetamine³⁻⁶, is repetitive, preservative behaviour that can be defined as the performance of an increasing rate of responses within a decreasing number of response categories⁶. In the rat, this is generally manifest as repetitive sniffing and motor movements, with repetitive licking and gnawing at higher drug levels. Although the mechanisms

controlling stereotyped behaviour are primarily dopaminergic, there are other modulatory influences, for example cholinergic and noradrenergic^{7,8}. Apomorphine is thought to act by direct stimulation of dopamine receptors in the telencephalon^{9,10}. There is some evidence that serotonergic mechanisms may be involved in stereotypy, because the integrity of the raphe nuclei is important for the expression of the stereotypy response produced by apomorphine and other agents¹¹. We have found that reductions in brain serotonin (5-HT) produced by diet result in decreased stereotypy after apomorphine administration.

Weanling, male Sprague-Dawley rats were housed at six per cage and given free access to food and water. Half of the rats were fed a corn-based diet containing Masa Harina as the source of protein and carbohydrate (low tryptophan rats) and the other half were fed an 18% casein diet (control rats). Each diet contained a vitamin supplement that supplied adequate amounts of niacin. The full details of the method and diets used have been described elsewhere^{2,12}. The rats were kept on these diets for at least 6 weeks and were rehoused in individual cages for at least 2 weeks before the start of the experiments.

On test days, rats were given doses (0.1-1.0 mg per kg, subcutaneously in the neck) of apomorphine HCl. Stereotyped behaviour (SB) was rated at 5-min intervals throughout the test session by experienced observers. The rating system was as follows: 0 = normal behaviour, inactive; 1 = normal behaviour, active; 2 = bursts of stereotyped sniffing, rearing or locomotion; 3 = continuous stereotyped sniffing, rearing or locomotion over a wide area of the cage; 4 = continuous stereotyped sniffing in one part of the cage; 5 = continuous SB with intermittent licking and gnawing; 6 = continuous SB with continuous licking and gnawing¹³. To assess differences between groups in the intensity of stereotypy, each rat's median score was determined, and pooled scores were analysed using the information statistic^{14,15}. To assess differences between groups in the duration of stereotypy, each rat's rating for the final 5 min of the test was evaluated (stereotypy or no stereotypy), and pooled scores were then analysed using the information statistic.

Table 1 Number of low tryptophan or control rats showing normal behaviour or stereotyped behaviour at the final rating period of the test

Dose of apomorphine (mg per kg, s.c.)	Diet group	No. of rats showing normal behaviour	No. of rats showing stereotyped behaviour
0.1	LT	9	1
	C	5	5
0.2	LT	8	2
	C	2	8
0.5	LT	6	4
	C	1	9
1.0	LT	8	1
	C	3	7

LT, low tryptophan; C, control. At each dose of apomorphine, the differences between the two groups are significant at least at the $P < 0.05$ level.

The apomorphine-induced stereotypy of low tryptophan rats was significantly attenuated compared with control rats (Table 1). In addition, at the 0.2-mg per kg dose of apomorphine, the low tryptophan rats showed less intense stereotypy than control rats (Fig. 1). These differences in intensity and duration of stereotypy between the two diet groups were abolished by pretreatment with L-tryptophan at 50.0 mg per kg, indicating that the reduction in stereotypy shown by low tryptophan rats was mediated by the serotonergic system.

After the behavioural experiment, a biochemical study was conducted to ensure that low tryptophan rats had decreased levels of brain 5-HT. Rats were decapitated at between 1000 h and 1200 h and brains were removed quickly, bisected midsagittally and frozen on dry ice. One half of each brain was assayed for serotonin¹⁶⁻¹⁸. Our results confirmed reports that

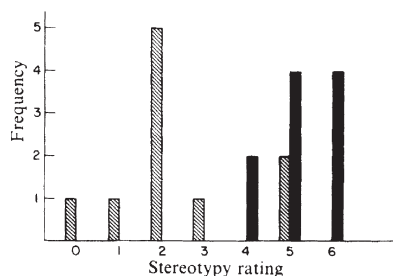


Fig. 1 Stereotypy after administration of apomorphine (0.2 mg per kg, given subcutaneously) in low tryptophan (hatched columns) and control (black columns) rats. Categories of stereotypy rating are on the abscissa. The frequency of each rating is summed over the test session.

rats maintained on a low tryptophan diet (corn diet) for 5 weeks have lower levels of brain 5-HT than rats maintained on a control diet (18% casein diet)^{1,2}: $588 \pm 31 \text{ ng g}^{-1}$ compared with $675 \pm 19 \text{ ng g}^{-1}$ (means $\pm$ s.e., significant at the $P < 0.05$ level ($t = 2.41$, Student t test)).

In experiments which replicated and extended the findings of the first study, we investigated the effects of a novel 5-HT agonist MK-212 and of quipazine on apomorphine-induced stereotypy in low tryptophan rats¹⁹⁻²¹. Again, the differences between the two groups in intensity and duration of stereotypy were abolished when the low tryptophan rats were treated with either MK-212 (3.0 mg per kg, intraperitoneally) or quipazine (5.0 mg per kg, intraperitoneally).

Finally, we investigated the effects of low tryptophan diet on another response thought to be mediated, at least in part, by dopaminergic mechanisms—psychomotor stimulant-induced hypothermia^{22,23}. The pattern of results for body temperature was similar to that obtained for stereotypy, in that low tryptophan rats showed a decrease in duration of hypothermic response after intraperitoneal administration of apomorphine at 5 mg per kg compared with control rats ($F = 6.75$; d.f. 1, 16; $P < 0.025$). Although the mean final hypothermic response under saline was the same for both diet groups (both $+0.2^\circ\text{C}$), the apomorphine-treated control rats showed a significantly greater decrease in body temperature compared with the apomorphine-treated low tryptophan rats (means -1.3 and -0.1°C , respectively). These results indicate that changes in brain serotonin can affect drug-induced hypothermia.

In conclusion, it seems that the decreased stereotypy response is associated with the decreased levels of brain 5-HT of rats reared on a low tryptophan diet. These findings show that while stereotyped behaviour is primarily mediated by dopaminergic (DA) mechanisms, it can also be modulated by other neurotransmitters, including 5-HT. This DA-5-HT interaction has implications for models of psychosis based on stereotypy²⁴. Further, this set of experiments demonstrates that diet can alter both neurotransmitter levels and behaviour.

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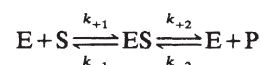
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Frequency-dependent selection due to kinetic differences between allozymes

ENZYME polymorphisms detectable by gel electrophoresis are common in populations of animals, plants and micro-organisms¹⁻³. Often the enzymes coded for by alternative alleles differ in their kinetic properties^{4,5}. Although the causes of enzyme polymorphism are much disputed⁵⁻⁷, there has been no systematic attempt to examine the possible selective consequences of kinetic differences between allozymes. We report here a theoretical study suggesting that in some circumstances kinetic differences lead to frequency-dependent selection, which is potentially capable of maintaining balanced polymorphism.

We consider first the simplest possible model of an enzymatically catalysed reaction



where E, S and P represent enzyme, substrate and product, respectively, and k_{+1} , k_{+2} , k_{-1} and k_{-2} are velocity constants. If steady-state conditions prevail, and the product is not accumulating, the velocity of the reaction is

$$v = \frac{V_{\max} [S]}{K_m + [S]} \quad (1)$$

where $V_{\max} = k_{+2} [E]$ and $K_m = (k_{-1} + k_{+2})/k_{+1}$. The equilibrium constant of the reaction, which is independent of the enzyme, is

$$K_{eq} = \frac{k_{+1} k_{+2}}{k_{-1} k_{-2}} \quad (2)$$

If the concentration of enzyme remains constant, the only way that $V_{\max}$ can be increased is by an increase in k_{+2} . In most circumstances, this will increase K_m (a compensating decrease in k_{-1} is unlikely because k_{+2} and k_{-1} both reflect the stability of the ES complex, and a compensating increase in k_{+1} is unlikely because of the constraint imposed by equation (2)). Thus, if there are two allozymes differing in their kinetic constants and one of them (A) has a relatively high $V_{\max}$ (represented as V_H), it is also likely to have a relatively high K_m (K_H). The lower constants of the other allozyme (a) can be represented as V_L and K_L .

This theoretical prediction is supported by the available experimental observations on the kinetic constants of alterna-

tive allozymes (Fig. 1). Although the data are sparse, they demonstrate a clear tendency for the values of $V_{\max}$ and K_m to vary together. This being so, at a given concentration of substrate, $[S]$, the ratio of the velocities generated by the two allozymes will be

$$R = \frac{v_A}{v_a} = \frac{V_H(K_L + [S])}{V_L(K_H + [S])} \quad (3)$$

When $[S]$ is very large relative to the values of K_m , this approximates to

$$R' = \frac{V_H}{V_L}$$

However, when $[S]$ is very small relative to the values of K_m , it approximates to

$$R'' = \frac{V_H K_L}{V_L K_H}$$

which is necessarily smaller than R' . At intermediate concentrations of S , the value of R lies between these two extremes. Thus, the relative velocity of the reaction catalysed by A is greater at high substrate concentrations than at low concentrations.

This conclusion is not restricted to a single-substrate reaction, nor to the unrealistic assumption that the reaction proceeds through only one intermediate. It follows from any system in which an increase in $V_{\max}$ is associated with an increase in K_m . It seems that the two constants can increase together even in complex systems (see Fig. 1 and, for formulae, ref. 8).

If the rate of the reaction contributes monotonically to the relative fitness of the organism (that is, if the reaction is rate limiting for some vital process, and if the selective value is a monotonically increasing function of velocity), then individuals with the A enzyme will be relatively fitter when substrate concentrations are high than when they are low.

To study the effect on selective values of varying the numbers and frequencies of individuals, it is convenient to assume that there is in the environment a substance, either the substrate or a precursor of it, whose effective rate of uptake by the organism is dictated by the velocity of the reaction. In this case, an increase in the number of organisms acts as a proportional increase in $[E]$,

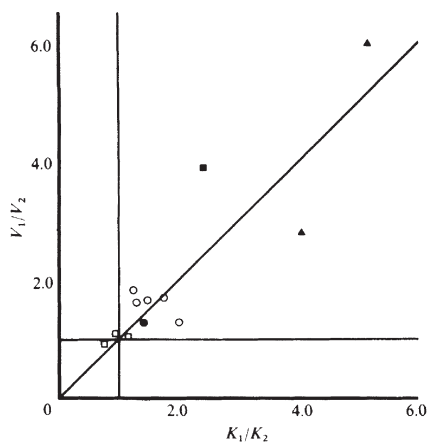


Fig. 1 Plot of relative maximum activities (V_1/V_2) against relative K_m values (K_1/K_2) in comparisons between allelic enzyme variants occurring in natural polymorphisms. The data come from five separate studies: $\circ$, ADH in *D. melanogaster* (D. Thatcher, unpublished data); $\square$, lactate dehydrogenase (LDH) in *Fundulus heteroclitus*²⁴; $\triangle$, penicillinase in *Bacillus licheniformis*²⁵; $\bullet$, glucose-6-phosphate dehydrogenase in man²⁶; $\blacksquare$, LDH in *Salmo gairdneri*²⁷. The several symbols for a single enzyme represent values using different experimental conditions, either various temperatures (LDH) or substrates (ADH and penicillinase), and they are not independent of each other. All the measurements were made on purified enzymes. Symbols in the upper right and lower left quadrants support the proposed relationship.

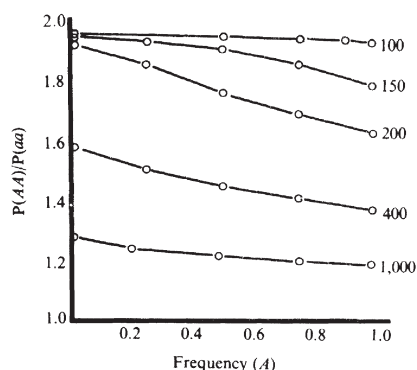


Fig. 2 The results of simulating the relative amount of substrate consumed, or product produced (P), during development per individual plotted against the frequency of allele A in a population with Hardy-Weinberg genotypic proportions. The model assumes kinetic constants approximating those found empirically for the ADH polymorphism in *D. melanogaster* (ref. 13 and D. Thatcher, unpublished). The values used are $A = Adh^F$: $V_{\max} = 900 \text{ pM min}^{-1}$, $K_m = 0.01 \text{ M}$; $a = Adh^S$: $V_{\max} = 450 \text{ pM min}^{-1}$, $K_m = 0.005 \text{ M}$. The heterozygote is assumed to contain only the two homodimers (because of the absence of information about the kinetic constants of the heterodimer). Development from egg to pupa is divided into 1,000 intervals of 10 min each. The amount of substrate (ethanol) consumed in each time interval is calculated by converting the rate equation (1) to a difference equation, and entering the appropriate values of substrate and enzyme concentrations. The enzyme concentrations per fly are assumed to be equal in flies of all genotypes and to increase linearly through the larval period to 100 pM per fly. The substrate concentration is increased at each interval by 0.0005 M (as if ethanol is being produced continuously by yeast in the food medium) and reduced by the amount consumed by the larvae during the previous interval. Five sets of simulations are shown that differ only by the number of larvae per vial (N), which is given for each set.

and therefore in $V_{\max}$ (without a concomitant increase in K_m). Simultaneous proportional increases in V_H and V_L will not alter the values of R' and R'' . However, if the supply of precursor or substrate is sufficiently limited, increases in V_H and V_L will accelerate its removal from the environment and will significantly reduce $[S]$. Consequently, they will shift the system away from the state represented by R' , towards that represented by R'' . In other words, an increase in the number of organisms will tend to reduce the selective value of A . The selection will be density dependent.

The selection will also be frequency dependent. An increase in the number of individuals carrying the more active A enzyme will have a greater effect on $[S]$ than an equivalent increase in the number of individuals carrying a . At high frequencies of A its selective value will be less than at low frequencies. This is frequency-dependent selection, which is potentially capable of maintaining a balanced polymorphism (see, for example, ref. 9), and follows directly from the difference between the two allozymes in their $V_{\max}$ and K_m . Figure 2 demonstrates that the frequency-dependent effect can be appreciable.

The existence of frequency-dependent selection in the conditions assumed leads us to enquire whether it provides a general explanation for selectively maintained enzyme polymorphisms. The generality of the assumptions must, therefore, be examined critically.

The first assumption is that the rate of reaction contributes monotonically to fitness. It implies that the reaction is rate limiting to some vital process, and thus excludes non-rate-limiting enzymes. However, although at any instant only one reaction in a pathway can be rate limiting, in a changing environment several or many may be successively so. There are, of course, situations in which an 'optimal' velocity can be exceeded, when overactivity is disadvantageous. In such situations, frequency-dependent selection of the balancing kind is less likely to occur, but it is not excluded.

The second assumption is that the removal of precursor or substrate from the environment is dictated by the velocity of the reaction. This again implies that the reaction is rate limiting, but also that it takes part in a pathway concerned with the uptake of an external substance. Enzymes catalysing reactions in deeply embedded internal pathways are not expected to be subject to this kind of frequency-dependent selection.

The third assumption is that the available supply of precursor or substrate is low enough to be significantly altered by changes in the rate of the reaction. This will certainly occur when there is ecological competition for the precursor or substrate (for example, as food), because such competition necessarily implies a shortage of the resource, but it can also occur in other circumstances when the resource is not crucial to the survival of the population. The depletion, if it is to produce frequency-dependent selection, must not be merely local to the individual; it must spread to affect others. This can occur most effectively when the substance is freely diffusible, and when the organisms are crowded in a fluid environment. The frequency-dependent selection is most likely to act on organisms that obtain their food in solution (as, for example, do many microorganisms, dipteran larvae and the roots of plants). It is less likely to act on animals that ingest particulate food.

The crucial question is whether unused precursor or substrate is returned to the environment in a form available to other organisms. Among herbivores and carnivores this return may take place through the faeces, but there will usually be a delay before the substance is once more available for consumption. Such a delay will certainly tend to destabilise the system.

Both intuition and simulation suggest that the frequency-dependent effect of substrate diminution will be strongest when the concentration of substrate approximates to the K_m of the enzyme. This situation is probably common. There seem to be long-term evolutionary pressures adjusting the K_m values of enzymes towards the average concentrations of their substrates¹⁰.

Finally, it is necessary to consider how often the frequency-dependent selection will actually, rather than potentially, maintain a polymorphism. Figure 2 shows a model derived from the kinetic constant of alcohol dehydrogenase (ADH) in *Drosophila melanogaster*, in which V_{max} and K_m seem to be roughly proportional to each other. Although the selective advantage of the more active enzyme falls as its frequency increases, it is nevertheless always superior to the less active enzyme. If a genuine balance is to be achieved there must be some additional disadvantage associated with a higher V_{max} . This disadvantage may be a disproportionate increase in K_m . The more active allelic enzyme is sometimes less stable (ADH¹¹⁻¹³ and xanthine dehydrogenase¹⁴ in *D. melanogaster*). Enzymes are often stabilised by the binding of substrate, so that when the concentration of substrate is low an enzyme with a higher K_m may be more liable to degradation.

The hypothesis that enzyme polymorphism can be a consequence of differences in kinetic parameters is attractive for several reasons. First, it offers an explanation of polymorphism that may be widely applicable. Second, the present model leads to several specific and testable predictions. For example, it predicts that rate-limiting enzymes should be more often polymorphic than non-rate-limiting, that enzymes with external substrates should be more often polymorphic than those intimately associated with the metabolic pathways of homeostatic organisms, and that enzymes with pervasive and freely diffusible substrates should be more often polymorphic than those with scarcer and less mobile substrates. There is already some support for these predictions. Regulatory enzymes, which are by implication rate limiting, have been claimed to have higher average levels of polymorphism than non-regulatory enzymes¹⁵ (but see ref. 16). Enzymes with external variable substrates are more polymorphic than those with internal constant substrates¹⁵. Invertebrates generally have higher levels of polymorphism than vertebrates², which have a better-developed internal homeostasis. Frequency-dependent selec-

tion has been reported to act on allozymic genotypes at two loci in *D. melanogaster* coding for enzymes with external substrates¹⁷⁻¹⁹.

The third attraction of the 'kinetic' hypothesis is that it offers a closer integration between the theories of enzymology, population genetics and ecology. We note, in this context, that selection for an increase in V_{max} resembles the *r*-selection of the ecologists²⁰, that selection for a decrease in K_m resembles *k*-selection, and that our model is mathematically similar to the models of inter- and intraspecific competition put forward by Stewart and Levin²¹ and by de Jong²². Ainsley²³ has suggested that kinetic differences in the ability of allelic enzymes to remove toxins can give rise to frequency-dependent selection, and his argument is similar in principle to our own.

In conclusion, it seems that biochemical differences (in V_{max} and K_m) between allelic variants of enzymes can, in some circumstances, lead directly to frequency- and density-dependent selection. Such differences may be responsible for maintaining a significant proportion of enzyme polymorphisms, particularly among organisms taking up dissolved food.

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Amino acid substitutions in two functional mutants of yeast alcohol dehydrogenase

A METHOD has been developed¹⁻³ for selecting mutants of the structural gene for the constitutive alcohol dehydrogenase of the yeast *Saccharomyces cerevisiae*. These mutations alter but do not destroy the function of the enzyme. We report here the amino acid substitutions in two of these mutants, and examine the probable effects of these substitutions on enzyme structure and function. This is possible since the amino acid sequence⁴, isozyme differences⁵ and possible subunit conformation⁶ of the protein are known.

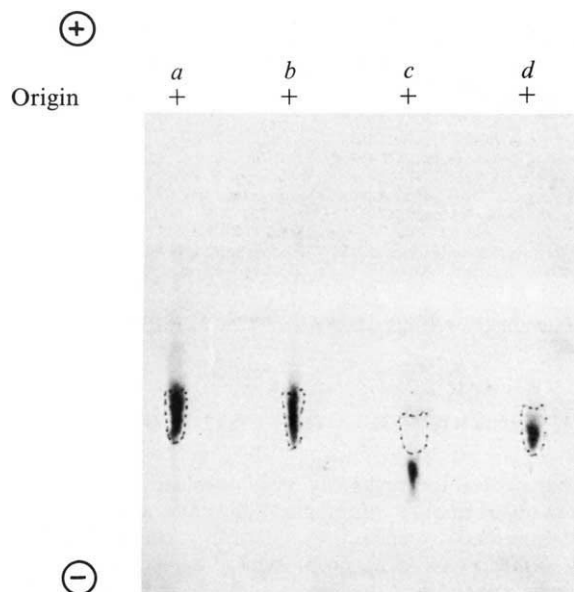


Fig. 1 Autoradiogram of neutral peptides separated by electrophoresis at pH 1.9. Fluorescent peptides are indicated by dashed lines. Samples are: *a*, mutant C-40; *b* and *d*, wild-type isozyme I; *c*, mutant S-AA-5.

The mutants were isolated from strain XW517-2D, a mating type. This strain carries a mitochondrial lesion rendering it incapable of aerobic respiration (cytoplasmic petite). In common with other petite strains, the only alcohol dehydrogenase found in the cytoplasm of this strain is the constitutive ADH-I, coded by the gene *adc*⁷. When grande strains of yeast, which are capable of aerobic respiration, are exposed to allyl alcohol, the majority of resistant mutants are ADH-negative^{1,8}. This prevents formation of the poisonous aldehyde acrolein from the allyl alcohol, which is by itself harmless. In contrast, petite allyl alcohol resistant mutants must continue to exhibit ADH activity, and their source of resistance has been shown in three cases^{2,3} to be due to shifts in the intracellular concentrations of NAD and NADH. A relative increase in the latter lowers the

equilibrium concentration of acrolein in the cell. Kinetic alterations observed in the mutant enzymes affect binding constants and cooperativity, and are compatible with the observed shifts³.

Two of these mutants, S-AA-5 and C-40, were analysed. Both mutant enzymes are more basic than the wild-type form, as deduced from electrophoretic mobilities. They also exhibit kinetic differences. The enzymes were purified using affinity chromatography on AMP-Sepharose and ion-exchange chromatography on DEAE-cellulose^{5,9}. Proteins were reduced and alkylated with iodo[¹⁴C]acetate in 6 M guanidine-HCl⁵. Digestion of 1 mg samples of the wild-type and mutant forms was carried out with trypsin. The mixture of peptides was separated on paper by multidimensional electrophoresis and chromatography¹⁰. The patterns were examined by autoradiography, fluorescence in UV light, and by staining with Cd-ninhydrin¹¹.

In both mutants, alterations were immediately apparent. There is only one radioactive neutral¹² tryptic peptide in ADH-I, and it is also the only fluorescent neutral peptide. It extends from position 39 to 59, including a histidine at position 44 (ref. 4). In S-AA-5, this peptide was absent, and instead two others were detected, a radioactive and a fluorescent fragment (Fig. 1). On staining with Cd-ninhydrin, the fluorescent peptide took a yellow colour, suggesting that it might now begin with Thr 45. This was confirmed by analysis of peptides isolated from 15 mg of the purified enzyme. The results, given in Table 1, show that the two new peptides comprise positions 39–44 (peptide 1) and positions 45–59 (peptide 2). The alteration in S-AA-5 was therefore His 44 → Arg.

The basic peptide beginning at position (315) in ADH-I, Ser-Pro-Ile-Lys⁴, was missing in mutant C-40, and replaced by two basic dipeptides, which on analysis (Table 1) proved to be Ser-Arg and Ile-Lys. The alteration in this mutant was therefore Pro(316) → Arg. In each mutant enzyme, the increased basicity and the extra tryptic cleavages are explained by the substitution.

The tertiary structure of the dimeric horse liver ADH has been determined to 2.4 Å resolution¹³. Although the yeast enzyme is a tetramer and has a highly dissimilar amino acid sequence, there is much evidence⁵ to suggest that the two proteins have a largely conserved subunit conformation. Therefore, it is reasonable to analyse the effects of the mutations in the yeast enzyme by examining equivalent positions in the horse protein.

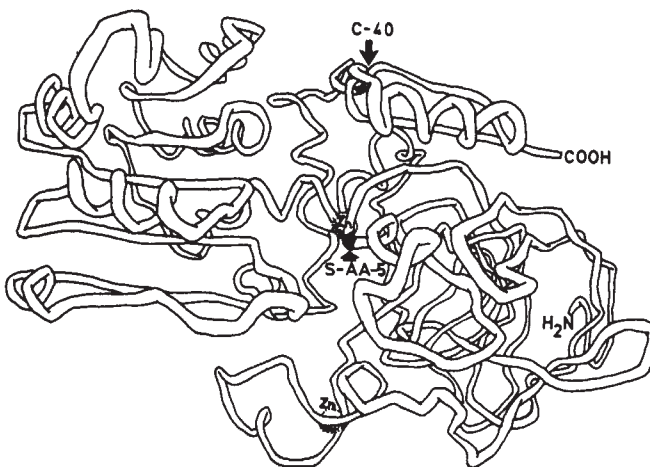


Fig. 2 Diagram of the polypeptide backbone in horse liver alcohol dehydrogenase. The positions corresponding to the exchanges in the mutants S-AA-5 and C-40 are indicated.

Of the two mutational changes, the His → Arg substitution in S-AA-5 has the most clearcut effect. The position is shown in Fig. 2, and corresponds to Arg 47 in the horse liver enzyme. This residue forms a salt linkage with the pyrophosphate moiety of the NAD^{13,14}. The substitution is thus one which would be expected to shift the binding characteristics of the cofactor away from those of the yeast and towards those of the liver enzyme.

Table 1 Data for the extra tryptic peptides in the mutants S-AA-5 and C-40

Mutant	S-AA-5		C-40	
	1 (radioactive)	2 (fluorescent)	1	2
Electrophoretic mobility at pH 6.5 ¹²	0	0	-0.63	-0.63
Recovery (%)	40	40	35	30
Composition				
Cys (Cm)	0.8 (1)	—	—	—
Asx	—	2.3 (2)	—	—
Thr	—	1.6 (2)	—	—
Ser	1.1 (1)	—	1.1 (1)	—
Pro	—	1.7 (2)	—	—
Gly	1.1 (1)	1.2 (1)	—	—
Ala	—	1.0 (1)	—	—
Val	0.9 (1)	—	—	—
Ile	—	—	—	0.9 (1)
Leu	—	2.3 (2)	—	—
Tyr	0.8 (1)	—	—	—
Trp	—	+ (2)	—	—
Lys	—	0.8 (1)	—	1.1 (1)
His	—	1.7 (2)	—	—
Arg	0.9 (1)	—	0.9 (1)	—
Total:	6	15	2	2
N-terminus	Tyr	Thr	Ser	Ile

As histidine may be less ionised than arginine at physiological pH, this linkage could be weaker in the yeast enzyme, resulting in a higher K_m . This is in fact the case. The K_m values for NAD for the yeast² and mammalian¹⁵ enzymes are 240 and about 10 mM respectively at pH 8.8. For NADH they are 140 and about 10 mM, respectively. In mutant S-AA-5, the K_m values for both NAD and NADH have been reduced to 60 mM, consistent with the expected effect of the substitution. As the reduction in K_m is not as great as in the mammalian enzyme, other substitutions must also play a part. This is compatible with the many differences observed in other residues involved in NAD binding¹⁴. This mutant also shows a loss of positive cooperativity in the reaction ethanol $\rightarrow$ acetaldehyde^{2,3}. The structural reason for this is not apparent, as the nature of the interactions in the quaternary structure are unknown.

The effect of mutant C-40 is more difficult to understand. The substitution Pro (316) $\rightarrow$ Arg occurs at a distance from both the active site and the known region of subunit interaction in the horse enzyme (see Fig. 2), yet this mutant shows alterations in both cooperativity and substrate binding. The K_m values for ethanol and acetaldehyde have been raised fivefold and twofold respectively, while those for NAD and NADH remain unchanged³. The differences appear significant even if the absolute values for the wild-type enzyme are not in agreement with those from other investigations^{16,17}.

The substitution occurs in the catalytic domain at a point corresponding to position 346 of the horse liver enzyme. This is at a turn between an extended structure along the inter-domain cleft, and a superficial strand of a β -pleated sheet region¹³. The substitution may influence the turn. It may also increase the spacing between the domains. With unaltered central inter-domain connections, this might restrict the pocket for the substrate and hence increase the K_m values. Alternatively, the substitution may affect interactions within the quaternary structure of the enzyme. The same region shows a number of differences in closely related isozymes (positions 178, (313), (337) and (338))^{4,5}. The lysine residue equivalent to Lys 228 in the horse liver enzyme, modification of which can increase the turnover number¹⁸, is also nearby, as well as a substitution (Ala 230 $\rightarrow$ Pro) in a variant form of human liver alcohol dehydrogenase¹⁹.

The two mutants analysed in this study are the first for which the amino acid substitutions have been determined. Many other independent allyl alcohol-resistant mutants have also been produced by selection. The present results show that such mutants do not necessarily represent changes at the same point in the molecule, not even when electrophoretic mobilities are similar (S-AA-5 showed 86% and C-40 87% of the mobility of the wild-type enzyme at pH 8.6). There is therefore strong reason to suppose that amino acid substitutions at additional positions will explain many of the other functional mutants of ADH-I, and that their determination will cast further light on the function of the molecule.

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Human complement C4 locus is duplicated on some chromosomes

WE have described genetic polymorphism of the fourth component of human complement (C4) and postulated that it was determined by an autosomal locus close to *HLA-B* on chromosome 6 (refs 1, 2). From familial and population studies of the electrophoretic C4 patterns, O'Neill *et al.*³ postulated that there are two C4 loci in all individuals, and that C4° alleles occur with high frequency at both. It was further shown that the blood groups Chido (Ch) and Rodgers (Rg) are closely associated with certain C4 types and indeed represent antigenic determinants on C4 molecules⁴, probably on the C4d part⁵. In this report we have used immunochemical methods to show that the C4 locus is definitively duplicated at least on some chromosomes. The implications are that the locus is either duplicated on all chromosomes and C4° alleles occur frequently at both, or alternatively, that the locus is duplicated on some chromosomes only.

High-voltage agarose electrophoresis and immunofixation/haemolytic assay reveal several different C4 phenotypes. Familial studies of the segregation of C4 phenotypes have shown that there are three main C4 haplotype products (not phenotypes): a slow four-band 'short' pattern (S), a fast four-band short pattern (F), and a seven-band 'long' pattern including all the S and F bands (F₁ or FS)². The most common of several rare haplotype products is a four-band short pattern (M) migrating slightly anodal to S. The S and M haplotypes are ordinarily Ch⁺, Rg⁻, the F haplotypes Ch⁻, Rg⁺, and the F₁ or FS haplotypes Ch⁺, Rg⁺ (O.B. *et al.*, in preparation).

We postulated that if the C4F₁ (Ch⁺, Rg⁺) haplotype product was a mixture of proteins coded for by two genes, it should be possible to separate the S (Ch⁺) protein from F (Rg⁺) with an appropriate antibody. If on the other hand, the haplotype product was coded for by one gene, an antibody directed at one part of the molecule should affect the electrophoretic migration rate of both the S (Ch⁺) and the F (Rg⁺) part of the haplotype product.

C4 typing of samples was carried out as described in detail previously². Ch and Rg typing of serum (plasma) was carried out by an inhibition assay of anti-Ch/Rg and C4 low ionic strength (LIS) coated red blood cells (RBC) using an antiglobulin reaction with IgG (R.N., in preparation). The anti-Ch serum used, serum OO, was from a patient recently transfused with several units of Ch⁺ whole blood. Using RBC coated with C4 from Ch⁺ serum in LIS medium, the direct agglutination titre in saline medium of this serum was 2,048, and more than 50,000 with an antiglobulin technique using pure anti-IgG. An IgG fraction of the OO serum was used in the studies described below. The serum was fractionated on a Sephadex G-200 column. The vacuum-concentrated IgG fraction gave a direct agglutinin titre exceeding 5,000 in saline against Ch⁺ C4-coated RBC, whereas the IgM fraction was negative.

Equal volumes of heparin plasma C4S (Ch⁺, Rg⁻) and the IgG fraction of serum OO, incubated for 1 h at 37°C, showed that the IgG fraction completely removed the electrophoretic C4S pattern from the plasma. The C4 pattern of C4F (Ch⁻, Rg⁺) did not change in the same conditions.

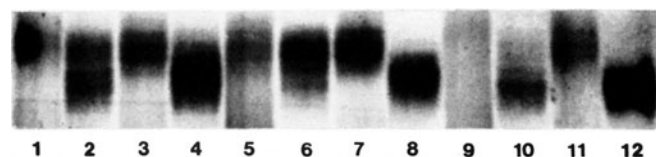


Fig. 1 Electrophoretic C4 patterns before and after absorption with IgG fraction of an anti-Ch antiserum. 1: Control C4F/F plasma sample; 2: F/S plasma sample; 3: F/S plasma sample absorbed; 4: FS/M plasma sample; 5: FS/M plasma sample absorbed; 6: FS/F plasma sample; 7: FS/F plasma sample absorbed; 8: M/S plasma sample; 9: M/S plasma sample absorbed; 10: FS/S plasma sample; 11: FS/S plasma sample absorbed; 12: S/S plasma sample control.

Figure 1 shows our main results. The individuals studied are members of families whose *C4/Ch/Rg* haplotypes are fully mapped from segregation in three generations. To summarise the results: The OO IgG fraction in this experiment absorbed S from S plasma, MS from MS plasma, S from F₁S plasma, S from F₁F plasma, S and M from F₁M plasma, and S from FS plasma. It did not absorb anything from F plasma. The control experiments exclude dilution and nonspecific degradation as causes of the observed results. We have therefore shown that the *C4* locus is indeed duplicated in some individuals, that is, on either one or both chromosomes in individuals segregating for the F₁ pattern. We therefore now recommend a change in nomenclature to haplotypes; a person homozygous for the two genes previously called F₁ would be of the haplotype *FS/FS* and a person heterozygous for one F₁ and one F or S would be either *FS/F* or *FS/S*.

The chromosomes not coding for long patterns may, however, still have only one *C4* locus, as the guinea pig seems to have, the only animal so far studied with similar techniques⁶. The postulate that all human chromosomes have two *C4* loci with a high frequency of *C4°* alleles³ remains unproved. This hypothesis can probably only be tested when it is possible to approach the problem on the DNA or RNA level. The present findings suggest that duplication/non-duplication of the *C4* locus may be present as a polymorphism of its own in humans. One may speculate, of course, on the possible forces behind a polymorphism of this kind. Could a duplicated *C4* locus represent an evolutionary advantage in humans, and thus represent a trait occurring increasingly frequently in the human race?

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Sequence of the 5'-end of *Strongylocentrotus purpuratus* H2b histone mRNA and its location within histone DNA

EXTENSIVE DNA sequence analysis of the cloned histone gene repeat unit from the sea urchin *Strongylocentrotus purpuratus* has identified the five histone protein coding sequences¹⁻³ (I.S., unpublished). R-loop and heteroduplex mapping strongly suggest that the sea urchin histone genes contain no intervening sequences⁴. In addition to the protein coding sequences the corresponding histone mRNAs each contain about 100 extra nucleotides^{4,5}. The location of these additional sequences in relation to the coding sequences is not certain. It is of interest to map the mRNA termini within the known DNA sequence because this information may help to identify transcriptional and translational control elements. Direct nucleotide sequence analysis of RNA can be carried out by enzymatic, base-specific cleavage of end-labelled RNAs^{6,7}. However, 5'-end labelling of mRNAs involves several enzymatic reactions ('decapping', dephosphorylation and phosphorylation) that require nuclease-free enzyme preparations⁸; furthermore, the RNA to be sequenced must be homogeneous. Indirect sequencing analysis of RNA can be obtained from its complementary cDNA. RNA has been sequenced in this way using a chain termination method with dideoxytriphosphates⁹, synthetic primers and purified mRNA templates¹⁰⁻¹². Alternatively, end-labelled restriction fragments have been used to prime synthesis of cDNA on homogeneous^{13,14} and partially purified^{15,16} mRNA templates followed by either chemical¹⁷ or enzymatic⁹ sequencing of the resulting cDNA. Our approach is similar and shows that prior purification of the RNA template is unnecessary. We present here the 5'-terminal sequence of *S. purpuratus* H2b mRNA, derived from purified H2b mRNA as well as from total polysomal RNA, using the chain termination method⁹ adapted for RNA sequencing. We also determine its location within the histone DNA sequence.

A short DNA restriction fragment, known to be located in the H2b protein coding region, was used as a primer for RNA-directed DNA synthesis. During an initial hybridisation step in which DNA/RNA hybridisation is favoured over DNA/DNA hybridisation¹⁸, the appropriate DNA strand binds to its complementary RNA sequence as a primer, and in this way selects the RNA template, which is then copied by reverse transcriptase in the presence of the four dideoxy chain terminators.

As shown in Fig. 1, we used as a primer a 51-base pair DNA fragment which is located in the NH₂-terminal end of the H2b coding region and is generated by *AluI* and *HaeIII* restriction endonucleases (New England Biolabs). The 3'-end of this priming DNA strand is located 10 bases downstream from the protein initiation codon. The fragment is short enough for the elongated chain-terminated DNA to be easily separated on the sequencing gels. This DNA fragment was obtained by endonuclease digestion of pRC39 (ref. 4), a plasmid which contains a completely sequenced *BamI/EcoRI* restriction fragment (1,016 base pairs) encompassing the H2b histone gene. The digestion products were separated on 8% (1/30 cross-linked) polyacrylamide gels. This homogeneous fragment was used to prime DNA synthesis by reverse transcriptase on both purified H2b mRNA and unpurified, total polysomal RNA as described below. In parallel with the RNA template we also used a DNA template, the *BamI/EcoRI* restriction fragment. The strands of this fragment were separated by electrophoresis on a 5% (1/60 cross-linked) polyacrylamide gel¹⁹ after denaturation in 50% dimethyl sulphoxide at 90 °C for 3 min (ref. 20). The faster-moving non-coding strand which has the same 5'-3' orientation as the mRNA was used as the template.

Five equivalents (10 pmol) of the double-stranded DNA primer fragment were denatured and hybridised to the

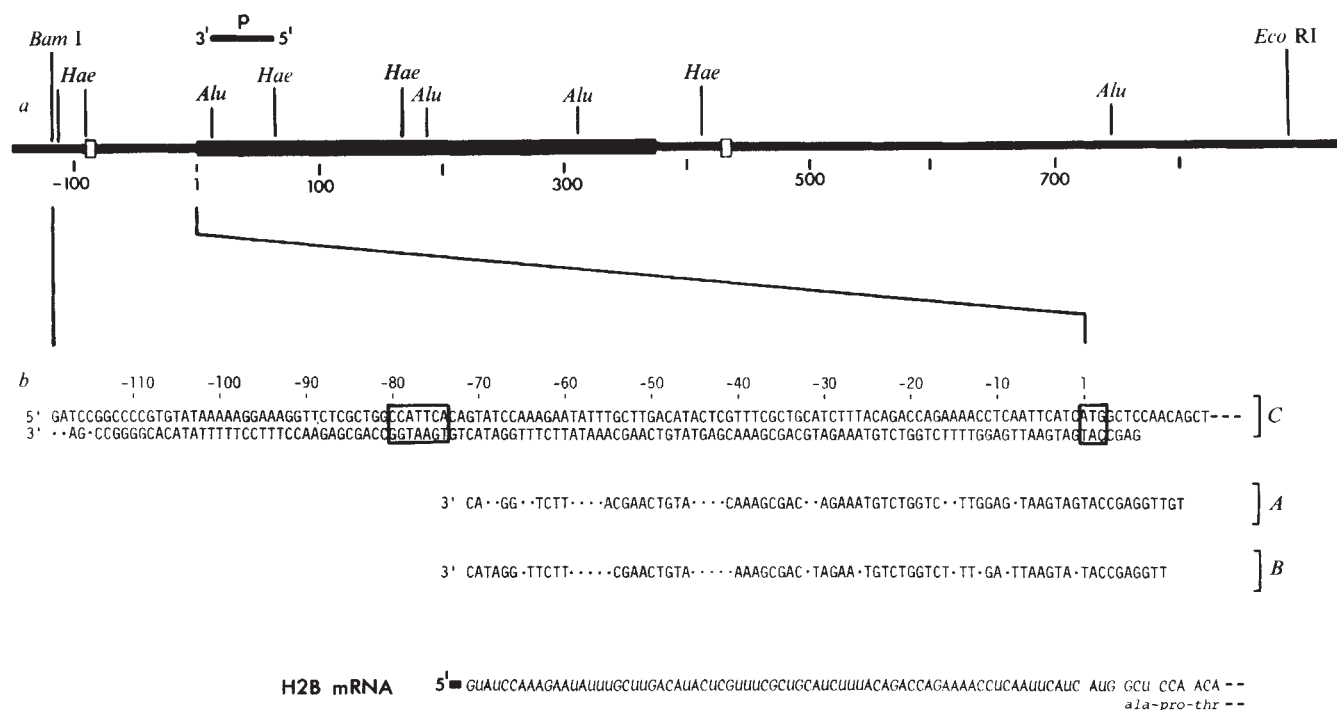


Fig.1 Nucleotide sequence of the 5'-end of histone H2b mRNA and its location within the DNA sequence of *S. purpuratus*. **a**, *AluI* and *HaeIII* restriction endonuclease map of the cloned *BamI*/*EcoRI* restriction fragment containing the H2b coding region (thicker horizontal line). Open boxes indicate the locations of nucleotides common to all sequenced *S. purpuratus* histone genes. Nucleotides are numbered by reference to the first nucleotide of the initiation codon. The DNA template for reverse transcriptase is the non-coding strand of the *BamI*/*EcoRI* fragment. The primer DNA (p) is the *AluI*/*HaeIII* fragment; its location and 3'-5' orientation is indicated. **b**, 5'-terminal nucleotide sequence of H2b mRNA (bottom line) deduced from the cDNA sequence of: (A) purified H2b mRNA; (B), polysomal RNA; (C), the non-coding DNA strand of the *BamI*/*EcoRI* restriction endonuclease fragment. Only the positively identified nucleotides are shown. In the double-stranded DNA sequence (C) the lower strand was determined in this experiment whereas the upper strand served as a template. The initiation codon and the common nucleotides PyCATTCPu are boxed. The 'cap' structure of the mRNA is indicated by the black box.

following: (1) One equivalent (2 pmol) purified H2b mRNA, assuming that the length of the mRNA is 500 bases^{4,5}. The mRNA was purified by hybridising total polysomal RNA extracted from early blastula stage *S. purpuratus* embryos to an excess of pRC39 DNA immobilised on cellulose as previously described²¹. The eluted RNA is of a single size, as judged by polyacrylamide gel electrophoresis, after 3'-end labelling with RNA ligase and ³²P-pCp (ref. 7 and I.S., unpublished). (2) One equivalent (2 pmol) H2b RNA in 125 µg total polysomal RNA assuming 0.25% of the polysomal RNA represents H2b mRNA. (3) One equivalent (2 pmol) single-stranded DNA of the *BamI*/*EcoRI* restriction fragment.

DNA/RNA hybridisation was carried out in 80% formamide, 2 × SSC at 50 °C for 2 h at primer and template concentrations of 1 µM and 0.2 µM, respectively. The conditions for DNA/DNA hybridisation were as described elsewhere⁹ except that the primer was separately denatured at 100 °C for 3 min before hybridisation. Denatured primer and single-stranded template were annealed at 68 °C for 30 min at concentrations of 0.8 µM and 0.16 µM, respectively.

The chain-terminated polymerisation reactions were carried out with reverse transcriptase as described in the legend to Fig. 2. Each set of reactions included a control mixture without dideoxy chain terminators¹³ to identify full-length transcripts and possible artefactual bands on the sequencing gel caused by nonspecific termination during the polymerisation reactions. The ³²P-labelled reaction products were separated on thin 7 M urea-8% polyacrylamide (1/30 cross-linked) gels²² and visualised by autoradiography.

Figure 2 shows autoradiographs of the sequencing gels. The three panels show the reverse transcriptase transcription products derived from purified DNA (*a*), polysomal RNA (*b*) and DNA templates (*c*). The sequences of the RNA-dependent transcripts (Figs 1*a*, *b*, 2*a*, *b*) are identical to the sequence derived from the DNA template. The sequenced portion of the 5'-end of the H2b mRNA is therefore co-linear with the corresponding sequence of the cloned sea urchin histone genes³ and does not contain any nucleotide insertions or deletions. This

co-linearity extends beyond the nontranslated leader sequence, at least to nucleotide 64 of the protein coding region (data not shown). The sequence derived from the DNA template (Figs 1*b*, 2*c*) confirms the sequence data previously reported³.

Sequences derived from the RNA template are ambiguous at certain positions, perhaps related to secondary structure or site-specific degradation of the RNA. Such nonspecific stops do not occur on the DNA template. The single-stranded DNA template which has the same 5'-3' orientation as the H2b mRNA was sequenced in parallel with the mRNA template. This side by side sequencing facilitates the determination of bases in these ambiguous positions. The DNA template, which is longer than the homologous mRNA, also serves as a control for nonspecific termination (see below). It is not necessary to determine beforehand the orientation (coding or non-coding) of the two separated DNA strands. They can both be used as templates in separate reactions and only the sequence derived from the non-coding strand serves as a reference.

It is interesting that the DNA sequence derived from purified H2b mRNA contains more nonspecific termination events than that derived from total polysomal RNA. These nonspecific stops are clustered (Figs 1*b*, 2*a*, *b*) and might be a result of secondary structure in the RNA template which is more pronounced in purified RNA.

Whereas the first 20 nucleotides of the RNA-dependent transcript can be read unambiguously, the first 4 nucleotides of the reaction with the DNA template are unreadable. They are probably obscured by internally labelled primer molecules. In the RNA-dependent reactions the primer is not labelled because of the presence of actinomycin D. As the purified H2b mRNA was isolated by hybridisation in vast DNA excess, contaminating DNA sequences homologous to the mRNA might be present and available as a template. Actinomycin D was thus included to ensure the exclusive use of RNA templates by reverse transcriptase.

Both RNA-dependent sequences (Fig. 2*a*, *b*) stop 71 bases upstream from the protein initiation codon (Fig. 1). Although we cannot exclude the possibility that reverse transcriptase stops

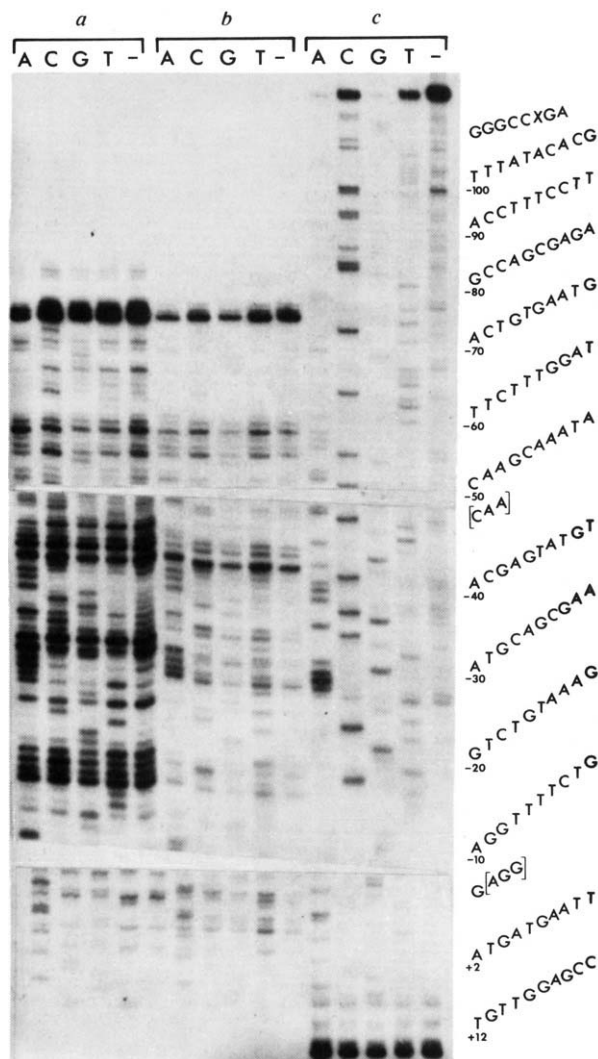


Fig. 2 Autoradiographs of sequencing gels showing the chain-terminated transcription products of the purified H2b mRNA (a), unpurified H2b mRNA (b) and the DNA (c) templates. Electrophoresis was on 8% (1/20 cross-linked) thin polyacrylamide gels²² containing 7 M urea, 100 mM Tris-borate, pH 8.3, 1 mM EDTA at 1,000 V, constant power. Sections of three gels, electrophoresed for different times, are shown, overlapping nucleotides are bracketed. The nucleotides are numbered by reference to the first nucleotide of the initiation codon. DNA/RNA hybrids were prepared as follows: 10 pmol primer DNA was dissolved in 9 μ l 90% formamide (MC/B), denatured for 5 min at 40 °C and transferred to silanised Eppendorf tubes containing 2 pmol of the dried H2b mRNA template. The solution was adjusted to 2 $\times$ SSC, 80% formamide by addition of 1 μ l 20 $\times$ SSC and hybridisation was carried out for 2 h at 50 °C. (1 $\times$ SSC = 0.15 M NaCl, 0.015 M sodium citrate). The formamide was diluted with 20 μ l 2 $\times$ SSC and the hybrids precipitated with 100 μ l ethanol, washed twice with 80% ethanol and dissolved in 12 μ l 1.66 $\times$ H buffer. (H = 50 mM NaCl, 34 mM Tris, pH 8.3, 6 mM MgCl₂, 5 mM dithiothreitol). DNA/DNA hybrids were prepared in sealed capillaries in a total volume of 12 μ l 1.66 $\times$ H buffer as described in the text. The RNA sequencing reactions were carried out in a total volume of 5.25 μ l in silanised Eppendorf tubes. Each reaction contained: 2 μ l hybrids in 1.66 $\times$ H buffer; 1 μ l dNTPs [500 μ M each of dATP, dCTP, dGTP (Sigma) and 85 μ M (100 Ci mmol)⁻¹ [α -³²P]-TTP (Amersham)]; 1 μ l 1 $\times$ H buffer containing 3 units reverse transcriptase from avian myeloblastosis virus (Life Sciences); 1 μ l of either 1 mM ddATP, 0.5 mM ddCTP, 0.5 mM ddGTP, 0.1 mM d(d)TTP (PL Biochemicals) or H₂O and 0.25 μ l actinomycin D (1 mg ml⁻¹) in 50% ethanol. In practice, a pre-mix containing hybrids, dNTPs and actinomycin D (Sigma) was added to the individual dideoxynucleotides, followed by the enzyme addition. Incubation was for 10 min at 42 °C in a final buffer concentration of 0.8 $\times$ H. To prevent nonspecific termination due to the low [α -³²P]TTP concentration, 1 μ l 0.5 mM TTP in 0.8 $\times$ H buffer was added after 10 min and incubation was continued for 10 min at 42 °C. The reaction mixtures were treated with 1 μ l 1 M NaOH at 100 °C for 3 min, chilled and neutralised with 1 μ l 1 M HCl. The DNA was finally precipitated with 150 μ l ethanol after addition of 0.5 μ g yeast tRNA (PL Biochemicals) in 45 μ l 2 M NH₄OAc and washed twice with ethanol. Samples were dissolved in 90% formamide, 1 mM EDTA, 0.05% xylene cyanol bromophenol blue, heated to 100 °C for 3 min, rapidly chilled and loaded on the gel. Samples containing DNA templates were prepared in the same way omitting actinomycin D and the final NaOH treatment.

because of a strong stop signal in the RNA itself, the enzyme does transcribe right through the same sequence on the corresponding DNA template. This sequence can be read clearly to the A of the *Bam*I site at position -116 (Fig. 2c). Therefore, it is very likely that the end of the sequences derived from the mRNA template represents the 5'-end of the H2b mRNA. The *S. purpuratus* histone mRNAs are capped with the structure 7mGpppX^mpYp (ref. 23). As we do not know if reverse transcriptase can copy the nucleotides at the X or Y positions of the 'cap' structure, we cannot conclude that G at position -71 is the very first base of the H2b mRNA.

The 5'-terminus of the H2b mRNA determined here maps next to a short nucleotide sequence which is common to all sequenced *S. purpuratus* histone genes³. This sequence, 5' PyCATTCPu 3' (boxed in Fig. 1), is found 50–70 bases upstream from the initiation codon for H2a, H2b, H3 and H4 and nowhere else in the sequenced part of the histone gene repeat unit. As the mature H2b mRNA does not contain this septanucleotide, it is probably not a translational control element. We note also that the 5'-end of the H4 mRNA has been tentatively located adjacent to the same sequence by matching H4 mRNA oligonucleotides to the known DNA sequence².

If we assume that the H2b mRNA is 500 nucleotides long, we can predict the location of its 3'-terminus within the DNA sequence. Interestingly, this prediction locates the 3'-terminus next to a nucleotide sequence [5' CAXGAAAGAXT 3' (boxed in Fig. 1A)] which is common to all three histone genes (H2a, H2b and H3) which have been sequenced downstream from the protein termination codon³. The function of these sequences in histone gene expression remains to be determined.

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Note added in proof: Recently we have formed DNA-RNA hybrids in the absence of formamide. This eliminated nonspecific chain termination events in sequencing the H2a, H3, H1 and H4 histone mRNA 5'-termini. Each of the 5'-termini coincides with the sequence 5'PyPyATTCPu3' in the genomic DNA.

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reviews

Still the Queen of Sciences?

Nevill Mott

Rutherford and Physics at the Turn of the Century. Edited by M. Bunge and W. R. Shea. Pp. 192. (McGill University Press: Montreal, 1979.) \$20.

APART from Rutherford, this book reminds the reader of the many others who were around in experimental physics at the turn of the century. In Cambridge, of course, there was J. J. Thomson, in France Becquerel, the Curies and Jean Perrin, in Germany, Röntgen, Hertz, Boltzmann and Planck, and in Holland, Zeeman and Lorentz. These men, building on the great achievements in mechanics and electromagnetism of the nineteenth century, made the discoveries which set in train the developments which led to the second explosion in our understanding, that in the decade of 1925 to 1935. This book, centered on Rutherford, is about how physicists looked at themselves and their discipline at that time, and provokes a comparison between the situation then and that in the *annus mirabilis* of quantum mechanics, and also with the present time.

The book is a series of essays by physicists and historians given on the occasion of the Rutherford Symposium at McGill University in October 1977, though at least one essay (by the late Norman Feather) had been published previously. The first, by Erwin Hiebert of Harvard, describes the state of physics at the turn of the century. First, he writes about the self-image of the physicists. They were supremely confident and physics to them was the queen of the sciences. In the nineteenth century, heat and electricity, once the domain of chemistry, had been added to physics, then called 'natural philosophy'. Now the science of the atoms, the classical domain of chemistry, was being added too. Its prestige, according to the author, was because of its generality, fundamental nature and theoretical pertinence for all the other sciences. This might have been argued at any time, but it was advocated far more openly and brazenly than ever before. This was not because of the impact on the outside world; the future of electrical technology was seen to be very promising, but apart from this the applications of chemistry to industry,



Photo: Otto Hahn

agriculture and war were overwhelmingly more important, in contrast to the situation today.

Apart from Einstein's Special Relativity and Planck's introduction of a quantum of radiation, the period is represented as the heyday of the experimental physicists, unexpected phenomena like X-rays, the electron and the radioactive transformation emerging one after another from their work. In this it contrasts with previous periods, with the massive theoretical achievement of Maxwell and before him Newton, and with the period from 1924-30 which saw the formulation of quantum mechanics. In this period theoretical work was inspired by an idea that did not prove successful, namely that of the mechanical Ether, which it was felt might through some singularities account for the electron and for other particles.

The experimental approach is seen as particularly English. An interesting essay by Niel Cameron (McGill) says that French thinkers, notably Pierre Duhem and Henri Poincaré, "regarded English physicists with a mixture of admiration and exasperation. They could find in them no trace of their own drive to achieve elegance, symmetry, completeness—only a passion for workable hypotheses, mechanical models and demonstrations that could

be given a readily comprehensible meaning. From the French point of view, the English had *no* theory, in the grand sense of the word, only a kind of inspired thrashing about".

The reviewer wonders if this is still a feature of English physics, in contrast with that of other communities. With frequent exchange and emigration particularly to the US there is probably less difference between one advanced country and another, and representatives of different ways of thought are to be found everywhere. Nevertheless, he remembers vividly the day when some young research workers burst into his room in the Cavendish excitedly, "Prof, come and see a moving dislocation", and showed him the little dark lines darting about on the screen of an electron microscope. "Jolly little beggars", Rutherford would have called them, and this was in the typical tradition of our country.

Perhaps also this was the end of the era when it was possible to doubt the physical existence of atoms and molecules. Ostwald was converted by cloud chamber photographs; the scientific historian Ernst Mach, founder of the Vienna Circle, perhaps never; but on the whole Rutherford's materialistic view prevailed because of its success. Rutherford, according to an essay by Stanley J. Jaki (Seton Hall), spoke about that reality with an intense commitment "as if he were defending the honour of a woman he loved". On being told by Eddington that electrons possibly were only mental concepts and had no real existence, Rutherford exclaimed, "Not exist? Not exist? Why I can see them as plainly as I see that spoon in front of me".

Most of the classic Rutherford stories are to be found in this book; and for the reviewer, there are some new ones. Of particular interest is the account of his move to McGill and the reasons for it. McGill's physics building was splendidly equipped; the donor of the institute, William Macdonald, had spared no expense. Rutherford expected—as he wrote to his fiancée, Mary Newton, in 1898—to be "practically boss man in the laboratory", but in fact this took him some time to achieve. His predecessor was Callendar, the man who revolutionised thermo-

metry, who left behind him a strong research group to carry on this kind of work. Callendar was a pupil of J. J. Thomson, an athlete, a mathematician, physicist, classicist and a fellow of the Royal Society, the first of J.J.'s pupils to rise so high. In short, when Callendar left McGill his colleagues naturally doubted that anyone so fine could be found to replace him. So at the end of his first year in McGill, Rutherford wrote to Mary Newton still waiting for him in New Zealand, "I am getting rather tired of people telling me how great a man Callendar was, but I always have the sense to agree. As a matter of fact, I don't quite class myself in the same order as Callendar, who was more an engineering type than a physicist, and who took more pride in making a piece of apparatus than in discovering a scientific truth". "This expression of snobbery"—as one essay puts it—"makes clear enough where Rutherford stood on the relative merits of pure and applied research".

And, as all the world knows, he had his way in McGill and achieved supreme success there (and incidentally the Nobel Prize for chemistry). Two things he had learned at the Cavendish had helped him. One was that one need not strive for accuracy in pioneering work. "In measuring the mechanical equivalent of heat (as did Callendar), "infinite pains are required"; in chronicling the activities of his "jolly little beggars" (ions), two-place accuracy would normally suffice. And the other was—how to form a research group. In fact, an interesting article by Lawrence Badach maintains that at McGill, Rutherford "laid the foundations of big science" in ways we would now take for granted, but perhaps not then: by the publicity he gave to his work, by the flow of research reports and publications in *Nature* and the *Philosophical Magazine*, and by the formation of research groups. Nothing succeeds like success, and it is perhaps true that at McGill Rutherford set the pattern which has carried physics to its present heights.

On reading this book, I am tempted to contrast the physics community of 1900 (± 5) with the Cavendish I first knew from 1927-33, and with the situation today. In 1902 one is almost surprised to find Rutherford writing to his mother from McGill, "I have to keep going, as there are always people on my track. I have to publish my work as rapidly as possible in order to keep in the race". This feature surely remains. In 1930, as now, all over the world in all branches of science there were and are enthusiastic research groups, enjoying their work, eager to publish and to stay in the race.

But there are now many more races. Rutherford's beloved nucleus, alas, can only be pursued with megabuck machines and a degree of theoretical understanding that eludes most scientists, even physicists in other specialities. Even a branch of the subject like solid-state physics has split into many divisions: semiconductors, metals, mechanical strength, plastics and amorphous materials, each with its journals and international conferences. In contrast to 1900 and 1930, the technological importance of physics, especially in microcircuits and nuclear energy, is enormous. The computer, a child of physics, enables the behaviour of matter to be predicted in a way that would have been inconceivable

two decades ago. Whether physics is still "Queen of the Sciences" as in 1900 and 1930 is more doubtful; some of the most startling developments in the last decades have been in biology, both pure and applied. However, most physicists will feel, rightly or wrongly, that an understanding of nature is only complete when expressed in the language of physics, and in that sense now as then we can believe that ours is the supreme discipline. □

Sir Nevill Mott was until 1971 Cavendish Professor of Physics at Cambridge. For his research work on the application of quantum mechanics to problems of solid-state physics, including non-crystalline semiconductors, he was awarded the Nobel Prize for physics in 1977.

What everyone needs to know about cancer immunology

Handbook of Cancer Immunology. Vol. 1: Basic Cancer-Related Immunology. Vol. 2: Cellular Escape from Immune Destruction. Vol. 3: Immune Status in Cancer Treatment and Prognosis (Part A). Vol. 4: Immune Status in Cancer Treatment and Prognosis (Part B). Vol. 5: Immunotherapy. Edited by H. Walters. Pp. 1867. (Garrland: New York and London, 1979.) \$37.50 each volume; \$165 the set.

SOME 25 years ago it became accepted that experimental animal tumours expressed neoantigens which elicited immune responses in the autochthonous host, or as its nearest approximation syngeneic hosts receiving transplanted tumour, and these responses could control tumour growth. Since then there has been an enormous growth of research into many aspects of the immunological recognition of malignant cells. Today this approach to cancer is at a stage where there is a need to review the concepts developed over the past years, rejecting those which no longer seem tenable and consolidating those deemed worthy of further study. Indeed critics will say the time has come for tumour immunology to substantiate the concept that host immune responses really do play a significant role in controlling cancer development.

Against this background, these five volumes attempt to provide an overview of basic developments in cancer immunology. This begins (volume 1) with a series of chapters on basic cancer immunobiology, and volume 2 deals exclusively with mechanisms by which tumours may escape immune destruction. Many of the individual

articles are excellent, providing short informative reviews of topics of particular interest to the authors. Nevertheless, one is left to draw one's own conclusions about the basic concepts of tumour immunology. For example, one would have expected volume 1 to provide a critical assessment of the relative merits of chemically-induced and virus-induced tumours compared with naturally arising animal tumours as models for human cancer. In fact this problem is not considered until later (volume 3) in reviewing the role of macrophages in resistance to cancer. The mechanisms by which tumours may escape immune destruction are considered in some considerable detail (volume 2) and this topic is further reviewed in volume 4. Altogether these articles provide a comprehensive survey of humoral factors such as circulating tumour antigen and antigen-antibody complexes which may interfere with cell-mediated immunity to tumour cells. But what of suppressor cells and factors released by these cells? Apart from their consideration in one particular animal system, there is no clear statement of opinions as to whether these cells do or do not play a decisive role in modulating immunity in tumour-bearing animals.

Turning next to human cancer, volumes 3 and 4 contain a series of articles presenting studies on specific types of malignant disease. The objective here is to review evidence that the selected human cancers do elicit immune responses in the patient. Here the selection of human tumour types for review and the order of presentation is somewhat puzzling. In reviewing studies with solid tumours and leukaemias the critical evaluation of methods for detecting cell-mediated and humoral immune responses is presented in volume 4, but this is preceded in volume 3 with chapters dealing with specific examples such as malignant melanoma and cancer of the

brain and bladder. Nevertheless, these chapters present accounts of the current status of research into the immunology of human cancer and it is refreshing to read critical appraisals in which the concepts developed from experimental animal tumour studies are tested. This includes an evaluation of the *in vitro* techniques for measuring cell-mediated immunity to human tumours, the conclusion being that these are not satisfactory for detecting or monitoring immune reactivity in cancer patients. Several of the chapters in volumes 3 and 4 also consider techniques for detecting antibody responses to human tumours. This approach came into vogue in the late 1960s, but with the introduction of more sophisticated assays which were thought to detect sensitised lymphocytes it fell out of fashion. Following recognition that the cell-mediated assays are more complex than originally thought, there has been a resurgence of interest in testing for anti-tumour antibodies. In this context it is recognised that antibody assays are particularly applicable for monitoring the isolation of tumour antigens in subcellular fractions. Unfortunately the biochemistry of tumour antigens is not dealt with in any great detail in these volumes, although the potential of the serological approaches in defining tumour antigens is illustrated in a review of melanoma associated antigens.

The final volume of the series sets out to review approaches for the immunological treatment of cancer. Surprisingly a variety of non-specific

agents such as cyclic nucleotides, polianions, levamisole and neuraminidase are considered, whereas the 'well-tried' agents, BCG and *C. parvum*, receive little attention. This approach may have been deliberate, as there are numerous reviews on cancer treatment with bacterial adjuvants. Nevertheless, this is to be regretted, as most of the clinical trials being carried out around the world have used either BCG or *C. parvum* and so a critical appraisal of this work should have been presented as a basis for the 'newer' approaches. This volume clearly emphasises how little is known of the inter-relationships of cell-mediated and humoral immune responses generated by a developing tumour so that it is still essentially impossible to devise rational approaches to the immunological treatment of cancer.

This is an ambitious series of volumes which probably succeeds as well as could be expected considering the vagaries of the individual contributors. The series as a whole provides an overview of cancer immunology but never really comes to grip with the basic problems. These concepts could have been presented in a much more economic fashion, which would have been more appealing to the uninitiated seeking guidance into the vast literature on cancer immunology.

R. W. Baldwin

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Virus diseases of trees and shrubs

Virus Diseases of Trees and Shrubs. By J. I. Cooper. Pp. 74. (Institute of Terrestrial Ecology/National Environment Research Council: London, 1979.) £3.

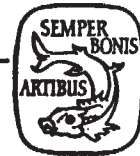
THIS book is a comprehensive collation and review of the virus, virus-like and mycoplasma diseases of a wide range of the exotic and indigenous trees and shrubs found in Britain. The book does not cover *Malus*, *Prunus* and *Pyrus* species because the author felt that these plants had been adequately covered in the literature on the virus diseases of fruit trees.

Many of the virus diseases discussed were studied in other countries but they are likely to occur or be introduced into the UK. Dr Cooper also describes the main methods of studying these diseases and determining their means of spread, deleterious effects and methods of control.

It is only in recent years that this important group of diseases of trees and shrubs has been studied. This book is a very useful summation of the available information, although there are a few conspicuous omissions, for example, virus diseases of *Chaenomeles*, *Cladrastis* and *Hibiscus* species. The book is clearly written and presented, and very well illustrated with a total of 65 colour and monochrome photographs, providing a very useful identification and reference guide to those involved in the study and diagnosis of tree and shrub diseases, or those involved in the propagation and production of woody plants. At the very reasonable price of £3, it is well within the reach of students and educational establishments and will do much to make horticulturalists, plant pathologists and plant health inspectors aware of the previously neglected, but important subject of the virus diseases of trees and shrubs.

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Biology of the intertidal zone

Biology of Intertidal Animals. By R. C. Newell. Third edition. Pp. 781. (Marine Ecological Surveys: Faversham, UK, 1979.) £22.

It is almost ten years since the first edition of Professor Newell's book appeared, and during those ten years there has been a revolution in the approach to the study of intertidal animals. Much of the natural history, the ways of life and overall ecology of the commoner intertidal animals was familiar and ably put together in the first edition. What has happened since is a reappraisal of intertidal invertebrate biology by zoologists interested in the physiological and biochemical strategies used by animals in adaptation to their particular niches. Fields of particular interest which may be mentioned are those of temperature acclimation, energy budgets and facultative anaerobiosis. This new edition contains much of the original descriptions of the basic natural history, feeding mechanisms, and so on. Although these parts are brought up to date, the arrangement of the chapters is different and reflects this change in emphasis which has occurred within the last few years.

The book is cast in four sections: distribution patterns, energy acquisition, metabolic energy expenditure, and a final chapter on energy budgets. Thus, although the first edition ended with a chapter on thermal stress and desiccation, the tolerance of environmental stresses generally is brought forward and the subject is properly discussed after the initial descriptions of the intertidal environment. This includes the recent work by Trueman and coworkers on burrowing. The descriptions of faunas of special habitats has been updated by the inclusion of the discussion of the work of Fenchel, Wieser and others on the role of bacteria and other microorganisms in the ecology of fine deposits. The chapter which follows integrates the many diverse factors influencing the distribution of intertidal organisms, emphasising the fact that the distribution and occurrence of any species is the resultant of the interplay of many factors. There is a useful introduction to Alderdice's approach to this problem illustrated by various examples, including Professor Newell's own recent work on *Ligia*. In the energy acquisition section there are useful discussions of the roles of dissolved nutrients, of microorganisms in the energy equations, and of deposit and filter feeding, and it is indicative

of the modern approach that these discussions precede the description of the actual feeding mechanisms. The concept of ration and factors affecting feeding rate and the energy budgets are properly discussed after the updated descriptions of feeding.

The third section concerns energy expenditure indicated by respiratory rate and includes discussions of the interesting work of de Zwaan, Bayne, Hockachka and others on facultative anaerobiosis and of the factors affecting the rate of oxygen uptake in aerobic conditions. The final chapter attempts an integration of the various adjustments available to organisms to maintain optimal fitness.

Review papers and some recent books concerned with thermal acclimation, anaerobic pathways, enzyme biochemistry and multi-factorial analy-

sis are not easy reading even for most advanced undergraduates. Where Professor Newell deserves congratulation in his new edition is in not only providing excellent summaries in themselves but in giving sufficient background for students for an appreciation of these exciting fields of research as an introduction for their further study. The book is very well produced in a larger format than the original and is to be recommended to all who wish to understand the biology of the intertidal zone. No library serving students' needs in the biological sciences should be without it.

R. P. Dales

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Rock mechanics

Experimental Rock Deformation: The Brittle Field. By M. S. Paterson. Pp. 254. (Springer: Berlin, Heidelberg and New York, 1978.) DM48; \$24.

LABORATORY experiments aimed at defining the mechanical characteristics of rocks under high confining pressure constitute a growth industry; people concerned with tectonic processes, earthquakes or the proper design of foundations for civil engineering projects are the customers. The subject divides naturally into the study of plastic deformation and the study of brittle fracture: the monograph under review is largely concerned with the second, but in spite of its title, it incorporates a valuable chapter on the brittle-ductile transition in rocks.

Professor Paterson, who has built up a flourishing school of rock mechanics in Canberra, has successfully set out to assemble a concise source-book. The bibliography is very extensive indeed and a scan through dates shows how modern a subject this is. David Griggs in California, building on the experimental genius of Percy Bridgman, was the father of modern rock mechanics, and his first citation dates back only to 1935. This bibliography will be of lasting value.

There are major differences between the mechanical behaviour of rocks and of metals, even brittle metals. Professor Paterson shows an impressive familiarity with the metallurgical literature and refers to it whenever it can illuminate a matter in hand; a special appendix is devoted to modern fracture mechanics, the metallurgists' creation. One special feature of rock behaviour

which is very clearly set out here is the linkage between incipient fracture and an anomalous enhanced dilatancy, an increase in specific volume. In the brittle-ductile transition, dilatancy again plays a major diagnostic role, and a distinction can be made between *cataclastic* flow (the microfracturing of rock followed by relative displacement of the fragments) and true crystal plasticity. The former process, but not the latter, is associated with measurable dilatancy. It seems that the dilatancy here discussed is much larger than the uniaxial microstrain preceding yield long studied by metallurgists.

Another unfamiliar subject well explained in the book is the stick-slip mechanism of frictional displacement, which has numerous implications for the behaviour of rock, both macroscopic and microscopic; presumably it serves to define the conditions of cataclastic flow. This chapter nicely supplements the long familiar treatment by Bowden and Tabor (*The Friction and Lubrication of Solids*; Clarendon/Oxford University Press: Oxford, 1950, 1964), who largely concern themselves with metallic friction. In this chapter and elsewhere in the book, Professor Paterson shows a necessary concern with the characteristics of the testing machine, stiffness in particular, which have a marked effect on the apparent fracture or flow behaviour of rock samples.

The book is exceptionally good value for money, and the reviewer now looks forward to completing his set in a few years' time with a companion volume on the plastic deformation of rocks.

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